FURTHER SEPARATIONS OF THE WATER-SOLUBLE COMPONENTS OF WHITE SPRUCE BARK

A Thesis

by

John Arthur Harpham
B.A. (Toronto)

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GENERAL INTRODUCTION

A knowledge of the individual chemical constituents of bark is important not only from an academic point of view, but is of vital interest to the economy of the wood pulp industry in general. For several years, a major investigation of the components of the bark of white spruce (Picea glauca) has been conducted at the Department of Industrial and Cellulose Chemistry at McGill University. Dr. Bishop (4, 5) instigated an examination of the little-known, water-soluble constituents, and his work has been continued by the author.

Methods designed to promote little or no chemical change were devised for the separation of the non-tannin materials, and a detailed examination of three fractions isolated was carried out. These three fractions were mixtures of partly-methylated glucosides, which had escaped hydrolysis by the naturally-occurring enzymes of the bark, and the aglycones seemed to be partly-methylated polyhydroxyphenols, based on C_{12} to C_{16} units. The results suggested that much of the original bark consisted of phenolic β-glucosides, which were less complex in structure than had hitherto been assumed. Valuable information was gained about the techniques that proved suitable for the investigation of the highly-colored, intractable, amorphous mixtures, with which the research was concerned.

HISTORICAL INTRODUCTION

Research carried out over many decades by many investigators has shown that the barks of plants consist of a complex mixture of substances, which vary in relative amount and in chemical detail with the species of bark examined. It was found convenient by Zellner, as reported by Segall (1), to classify the constituents into five broad groups according to their solubility. Groups 1 and 2 comprised the aliphatic oils, waxes, alcohols, acids, resin acids, terpenes, sterols and oxygenated derivatives that were soluble in ether. Group 3 was the water-insoluble, alcohol-soluble phlobaphenes; group 4 the tannins, glycosides and sugars that were soluble in both alcohol and water; and group 5 the water-soluble, alcohol-insoluble materials. Excellent reviews on bark and bark extractives were compiled by Kurth (2), Segall and Purves (1), Harwood (3, 4) and Bishop (4, 5), references (1) and (3) being of special importance for the water-insoluble extractives. These extractives, which together amount to 8% to 30% of most barks, were the subject of a recent thesis by Harwood (3) and needed no further discussion on the present occasion. A discussion of the problems arising from the large-scale disposal and utilization of bark was to be found in the Northeastern Wood Utilization Council's Conference on Bark (6).

Aqueous Extractives from White Spruce Bark

The research of Bishop (5) on the water-solubles, however, required detailed treatment, because it formed the background to the present research. Much of the previous work on the aqueous extracts merely listed the amounts of sugars, glycosides, polysaccharides, tannins and phlobaphenes present. Investigation of properties and structure was lacking in most cases.

Bishop's empirical fractionation of the water-soluble portion of white spruce bark, outlined in his Figs. I, II, III, IV, required little explanation. The bark was obtained directly from the woods, thus preventing any loss of extractives which might have occurred had the logs been "river-run". Throughout the investigation, precautions were taken to minimize chemical changes which might have resulted from laboratory conditions, i.e., high temperatures, or strong acids or bases, or biochemical causes, such as fermentation or other enzyme action, or mold growths. Quantitative results were sometimes difficult to obtain, because of the large quantities being handled, but every effort was made to attain this objective.

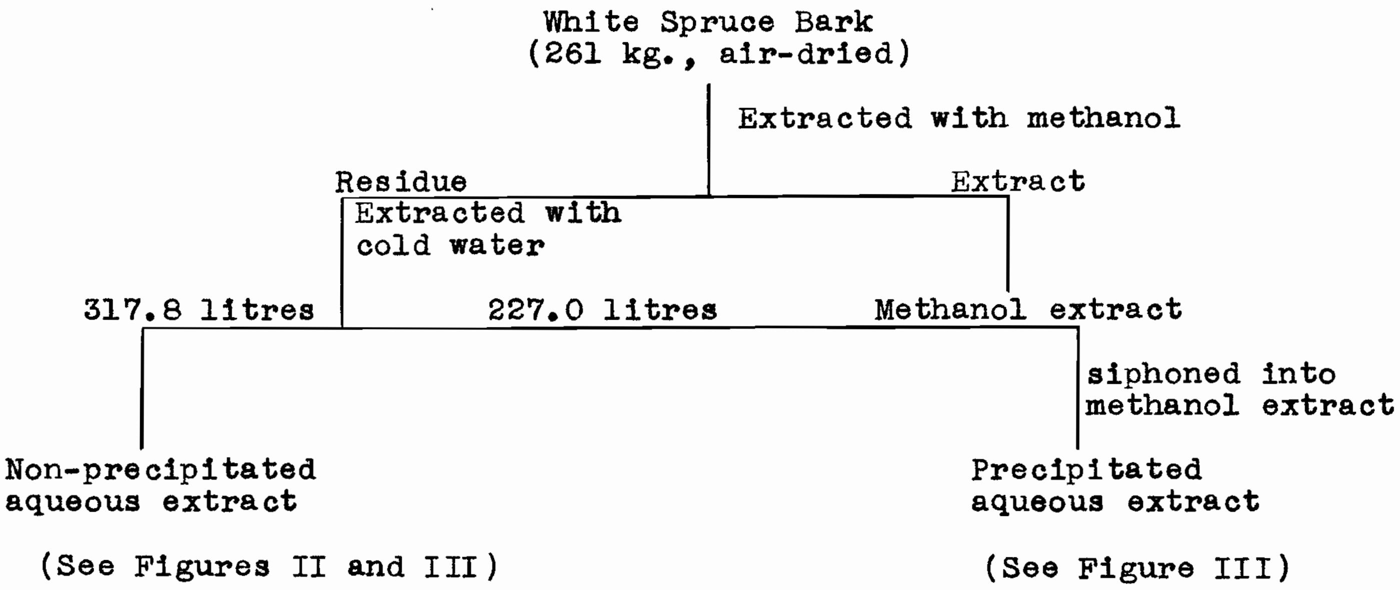
While the present research concerned only a further investigation of fraction 21 (Fig. IV), some of the results for each of the fractions obtained by Bishop have been summarized, in the interests of clarity, by tables reproduced from his thesis. Table I listed the yields of fractions from 261 kg. of air-dried spruce bark, while Table II gave the results of sugar analyses for various fractions. Both free and combined glucose, and some mannose were found to be present in many fractions, whereas galactose, fructose and rhamnose were absent. The negative results were in direct contrast to the findings of other workers (7, 8), on other species of bark.

After the various fractionations up to fraction 20, outlined in Figures I to IV, had been performed, neither concentration of the black aqueous liquor nor the addition of organic solvents caused further precipitations, and dialysis was carried out to separate the components of the mixture. In order to avoid chemical changes at high temperatures, a special continuous vacuum-dialyzing apparatus was constructed. Operated at a pressure of approximately 2 cm., dialysis was performed batchwise, until the total solids in the dialysates reached constant weights. The non-dialysable portion (fraction 21), constituting 23.8% of the bark by weight, was concentrated to roughly 50% total solids, and stored under toluene to prevent fermentation, pending further investigation.

The standard hide powder determination (9) showed the bulk of the tannin to be present in this fraction, making up 52.5% of the total solids. Some tannin material had dialyzed, since 8.34% of tannin was present in fraction 23. Total tannin determinations, in conjunction with small-scale extractions of bark conducted by Harwood (3), showed that only three-quarters of the tannin present in white spruce bark had

FIGURE I

Preliminary Fractionation of Cold-Water Extract (5)



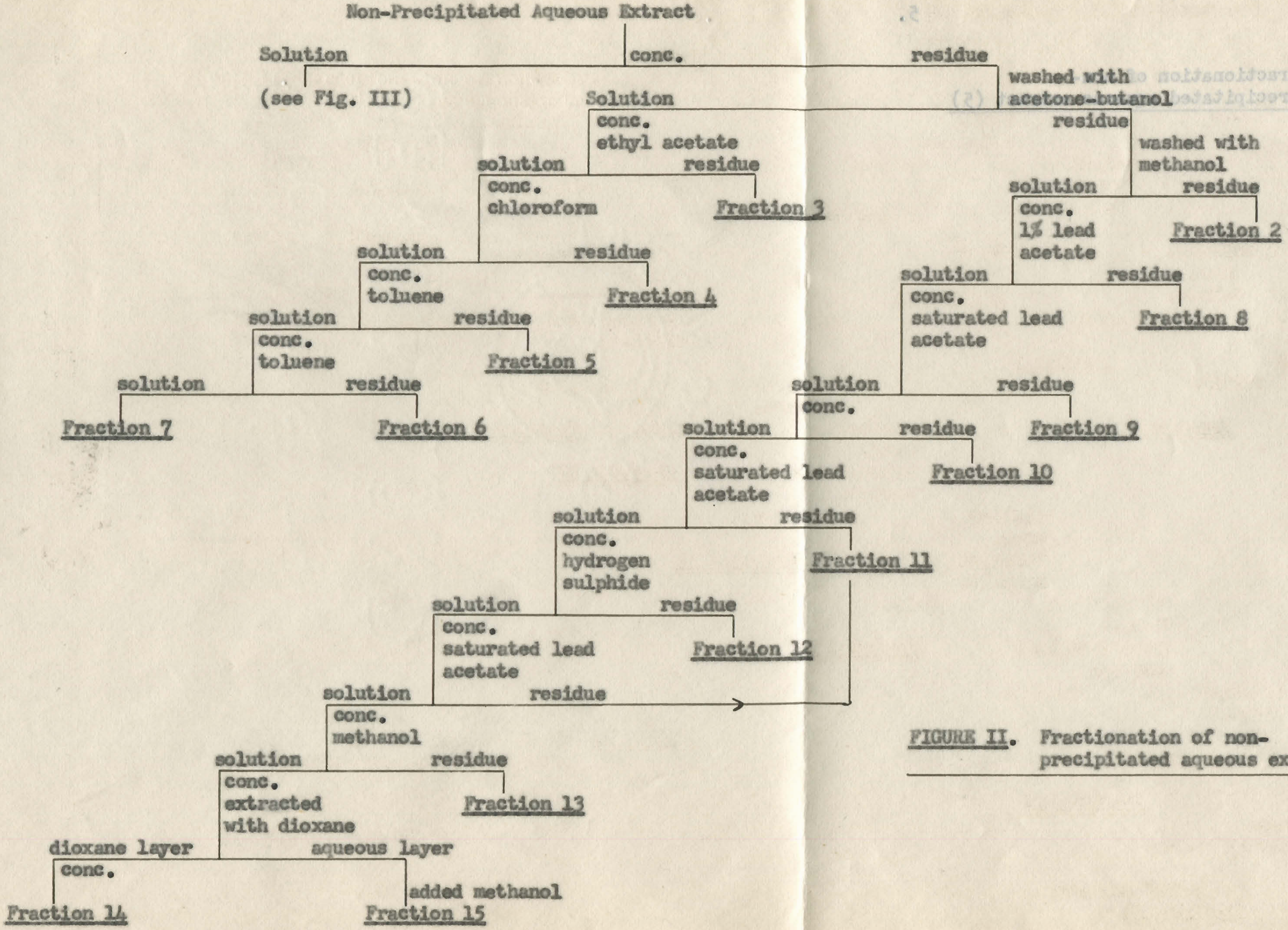


FIGURE II. Fractionation of non-precipitated aqueous extract (5)

FIGURE III

Fractionation of Precipitated and Non-Precipitated Aqueous Extracts (5) (Fractions 1 and 16 to 19)

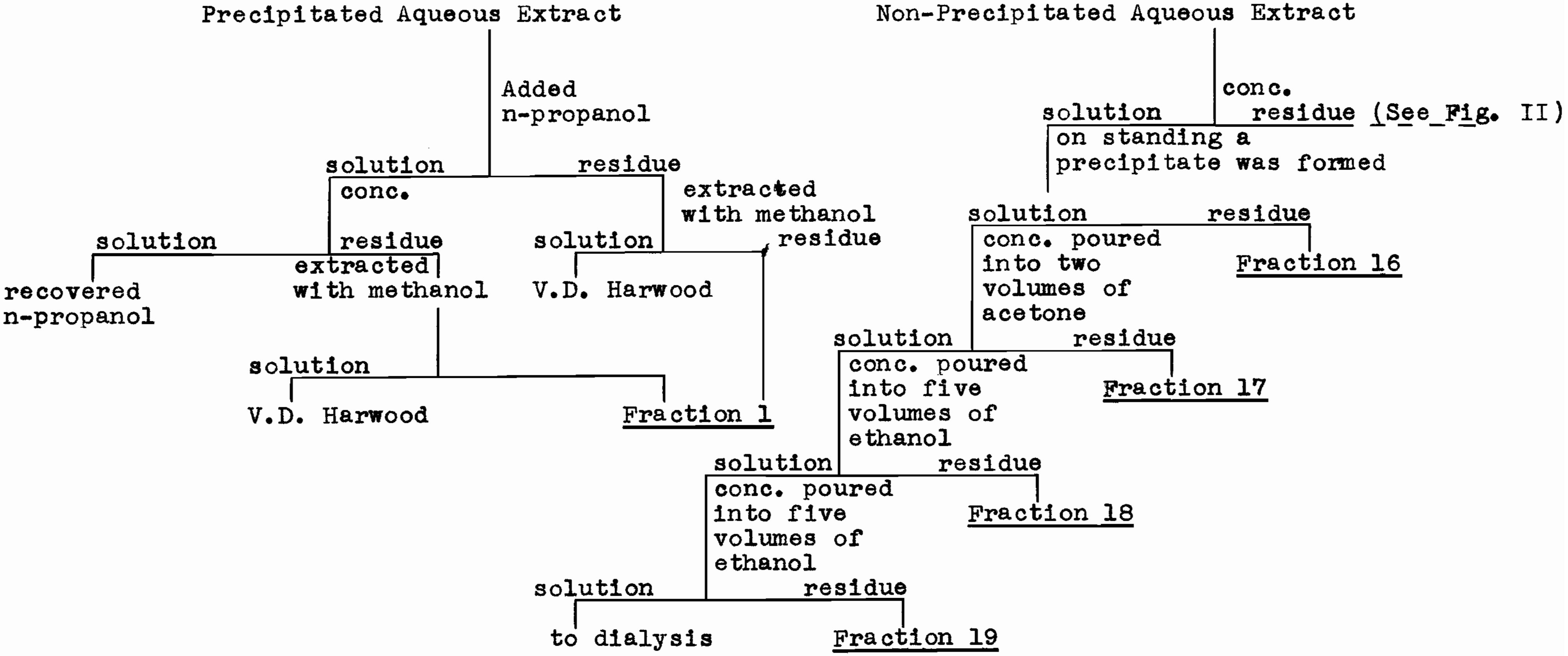


FIGURE IV

Fractionation of Hot-Water Extract and Methanol-Water Solubles (5) (Fractions 19 to 24)

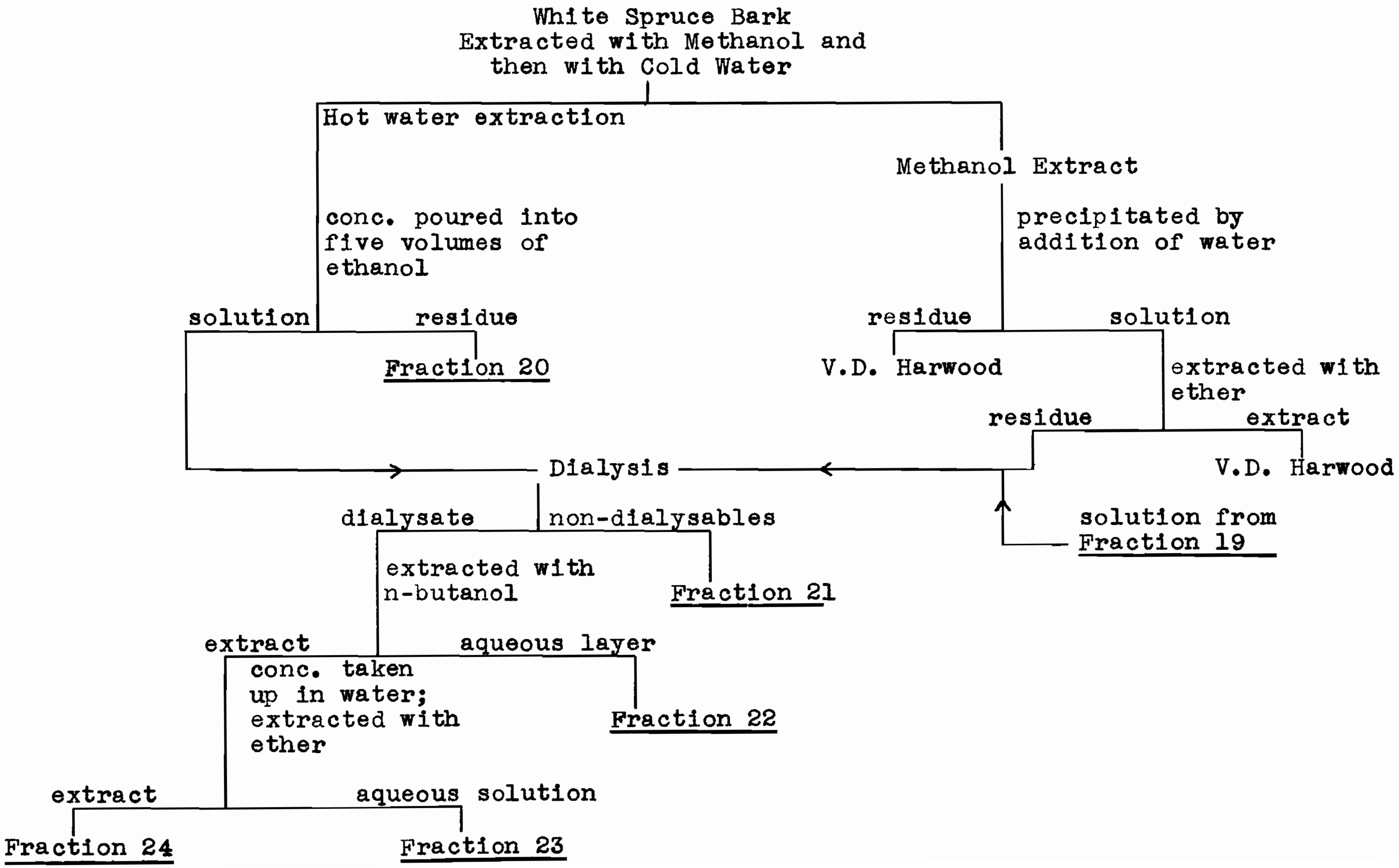


TABLE I

Fractionation of Water-Soluble Bark Constituents (5)

Number of Fraction	Weight in Grams	Percentage Based on Dry Weight of Bark
1	423.5	0.162
2	1259.2	0.482
3	4.5	0.002
4	176.1	0.067
5	10.5	0.004
6	1.0	...
7	...	...
8	175.0	0.067
9	150.6	0.057
10	20.1	0.008
11	126.4	0.048
12	3.0	0.001
13	109.7	0.042
14	51.5	0.019
15	445.1	0.170
16	263.4	0.107
17	635.2	0.242
18	449.0	0.173
19	165.0	0.063
20	1425.0	3.720
21	45100.0	23.8
22	927.5	0.54
23	276.4	0.16
24	42.6	0.024

TABLE II

Sugar Analyses on Various Fractions(a) (5)

Fraction	Free Reducing Material	Reducing Material After Hydrolysis	Time Required For Complete Hydrolysis (hours)(e)	Reducing Material After Fermentation(f)	Fermentable Sugars(f)	Pentoses
1(b)(f)	1.22	16.0	6	5.6	10.4	2.0
2(c)(f)	1.30	13.2	8	3.2	10.0	1.8
16	1.18	23.2	6	5.2	18.0	1.65
17	0.48	30.0	4	22.4	7.6	8.88
18	1.32	19.2	4	16.0	3.2	4.70
19(d)(f)	1.08	32.0	4	1.6	30.4	6.40
20	0.62	28.6	4	16.3	12.3	19.22
21	27.4(g)	54.0	4	0.0	54.0	1.55
22	47.6	76.0	3	1.73	74.27	2.07
23	20.0	19.7	3	1.08	18.92	0.93

(a) All results are % of total solids in the fraction. Mannose and fructose always absent unless otherwise noted.

(b) Mannose 10.2%. (c) Mannose 6.6%. (d) Mannose 6.4%.

(e) With 3% hydrochloric acid at 100°C. (f) All determined after hydrolysis.

(g) After standing for six months.

been removed by the large-scale methanol extraction. The good quality of this tannin was indicated by an increase in the shrinkage-point temperature (10) from 65° to 80°. Alkali fusions of the isolated crude tannins resulted in complex mixtures of phenolic compounds, from which a small yield of protocatechuic acid was isolated. All fusion products were qualitatively shown to contain phenolic groups. Qualitative color tests for tannins were all positive. The action of emulsin reduced the tannin content of fraction 21 from 52.5% to 39.0%.

When first isolated, the non-dialysable material was freely water-soluble, and its aqueous solution was non-reducing to alkaline copper reagent. Over a period of 6 months' storage, the water-solubility diminished to the extent that dilution with water caused the precipitation of 76% of the total solids, while 27.4% of copper-reducing material was present in the filtrate. Such an occurrence could only be explained by enzyme action, since acid hydrolysis at pH ca. 6 and room temperature was extremely unlikely. Acid hydrolysis increased the copper-reducing power (as glucose) to 54.0%, and caused the formation of an insoluble, phlobaphene-like solid. The pentose content was small, amounting to 1.55%. The complete destruction of copper-reducing power by fermentation, both before and after hydrolysis, despite the yield of apparent furfural, plus the fact that neither mannose nor fructose could be found, showed glucose to be the only sugar present.

The presence of glucosides of the β-configuration was proven by independent methods; emulsin increased the amount of copper-reducing material from 27.4% to 37.0%, and caused the deposition of a gummy precipitate similar to the one obtained on dilution with water; heating a portion of fraction 21 in aqueous alkali, and methylating the products, resulted in the formation and isolation of 2,3,4-trimethylglucosan <1,5>-β-<1,6>. The conditions used were specific for the production of glucosans from glucosides of a phenolic nature and having the β-configuration (11). A yield of the above-mentioned glucosan corresponding to 9.5% of the glucose in fraction 21 was isolated, but the percentage might have been even higher, as there was no way of determining whether or not the alkaline cleavage and methylation were quantitative.

The precipitates caused by emulsin, dilution with water, and acid hydrolysis, being dark brown, amorphous, water-insoluble, alcohol-soluble materials, had properties similar to phlobaphenes.

In order to investigate fraction 21 further, a 70-g. portion of the water-insoluble precipitate, after three reprecipitations, was hydrogenated in alcohol at 2850 p.s.i. and 250°, with a copper - chrome oxide catalyst. Neither free nor combined glucose was present in the amorphous material originally. After 26 hours, 2.0 gram moles of hydrogen had been absorbed, and the color of the alcoholic solution had changed from very dark brown to yellow. An 83% recovery of material from the hydrogenation indicated a loss of 17% as water or gaseous products. At 0.11 mm. pressure, 51% of the products boiled below 190°. By fractional distillation through a modified Vigreux column at both atmospheric and reduced pressures, the products were separated into four fractions. Fraction 1 consisted mainly of ethanol and water, plus traces of a volatile amine. Although a crude crystalline hydrochloride was isolated, the amount was not sufficient for positive identification. It was assumed to be dimethylamine, from similar work done by Gleason (12). Fraction 2 amounted to 22.4%, and after careful refractionation through a Cooke-Bower column, a product was isolated from it in a yield of 5.9%, which was positively identified as 1,2,4-butane triol. Fraction 3 (28.6%), after refractionation, yielded no solid derivatives, although analytical data for hydroxyl derivatives of cyclohexane and propylcyclohexane were obtained. The still residue, fraction 4, represented 32% of the starting material. It consisted of a light brown solid fusing at 58°-70°, soluble in most organic solvents, but insoluble in water. Its average molecular weight (Rast method) was 437, and an analysis for $C_{27}H_{43}O_{4.5}$ indicated a mixture of a cyclic, highly condensed nature. While the oxygen was probably present as ether linkages, no identified cleavage products were obtained by the use of hydrogen iodide. Although the identification of the hydrogenation products was incomplete, it was tentatively concluded that the amorphous material was of relatively low molecular weight, having C_4 units attached to cyclohexane groups.

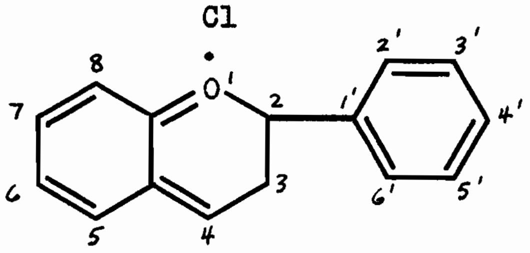
To sum up, the work of Bishop showed clearly, and for the first time, that the large non-dialysable fraction of the water-soluble materials consisted overwhelmingly of phenolic glucosides. A survey of what was known of the naturally-occurring types of these compounds was then undertaken.

Naturally-Occurring Glycosides

Representatives of nearly every class of organic compound have occurred as glycosides in vegetable material, chiefly in the fruit, bark and roots. Glycosides of alcohols, phenols, acids - to mention but a few, have been isolated and identified. While glucose made up the carbohydrate portion of these compounds in most cases, hexosides derived from galactose, mannose and fructose, pentosides derived from arabinose and xylose, and methyl pentosides derived mainly from rhamnose, were also present. The book by Armstrong (13) tabulated the origin and physical properties of the well-defined, crystalline glycosides.

The present discussion was restricted to bark glycosides and compounds related to their aglycones. These glycosides included the natural coloring pigments (anthocyanins, flavones, and flavone derivatives), and tannins, in so far as tannins appeared similar to the natural coloring pigments.

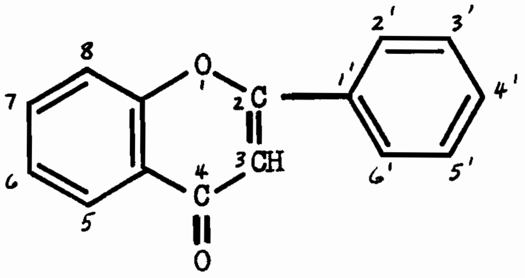
The simplest structural unit of the anthocyanins has been represented by flavylium chloride or 2-phenylbenzopyrylium chloride (I). All members of this group were to be regarded as polyhydroxy-2-phenylbenzopyrylium salts, hydroxyl substitution



I

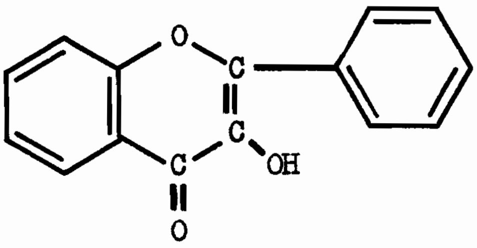
taking place in the 3,5,7 positions, and in one or more of the 3',4',5' positions. All naturally-occurring compounds were glycosides, with the sugar residues attached to the 3- or 3,5-hydroxyl groups, i.e. (a) the 3-monoglucosides and 3-monogalactosides; (b) the 3-rhamnosides and 3-pentosides; (c) the 3-biosides; (d) the 3,5-diglycosides; and (e) the acylated anthocyanins; (14). While these glycosides occurred mainly in flowers, Roach (15) stated that colored substances of apparently the same class were precipitated by lead salts from the alcohol extract of apple bark. The works of Robinson, Karrer and Willstätter were classics in the anthocyanin field, and a review of Willstätter's researches has been published by Robinson (16).

The basic structure of the flavones was given by II, which was flavone itself. Other flavone pigments were derived by attaching hydroxyl groups to one or more of the 3, 5, 6 and

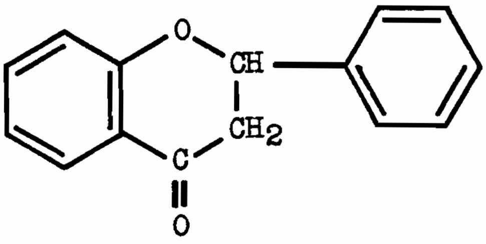


II

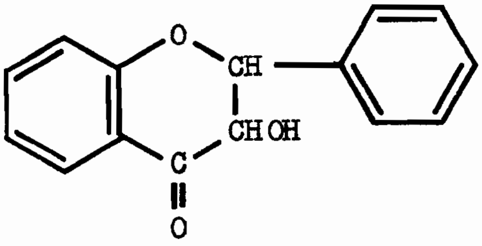
7 positions, and to one or more of the 3', 4', 5' positions. Flavonol (III), flavanone (IV) and flavanol (V) were also to be considered as basic units. They have occurred naturally in



III



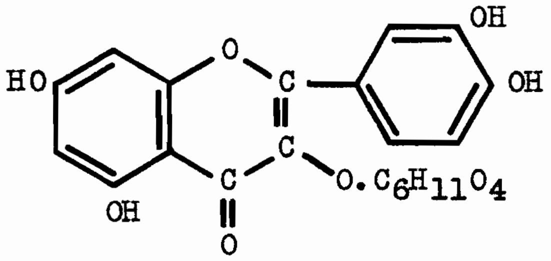
IV



V

combination with rhamnose or glucose as glycosides, sometimes uncombined, and frequently also associated with tannins.

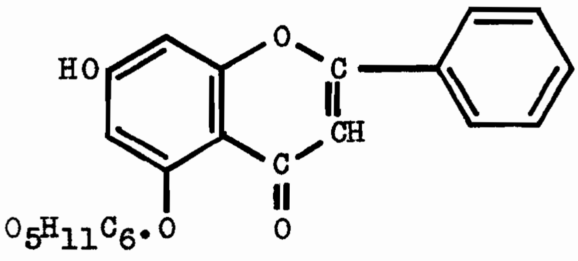
Oak bark was the main source of quercitrin (VI), the



VI

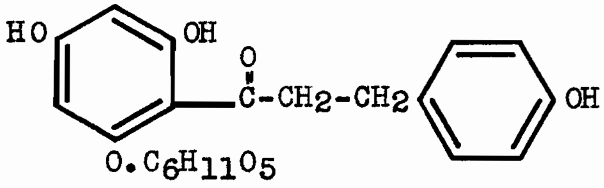
rhamnoside of quercetin. An unusual occurrence was von Lippmann's discovery of the crystalline glycoside on the bark of an oak tree below a deep gash. Purification consisted of treating the solution with a little lead acetate and decolorizing charcoal, dialysis, and crystallization from hot water (17).

Toringin, or chrysin 5-glucoside, (VII) has been reported in the bark of Docyniopsis tschonoski (18).



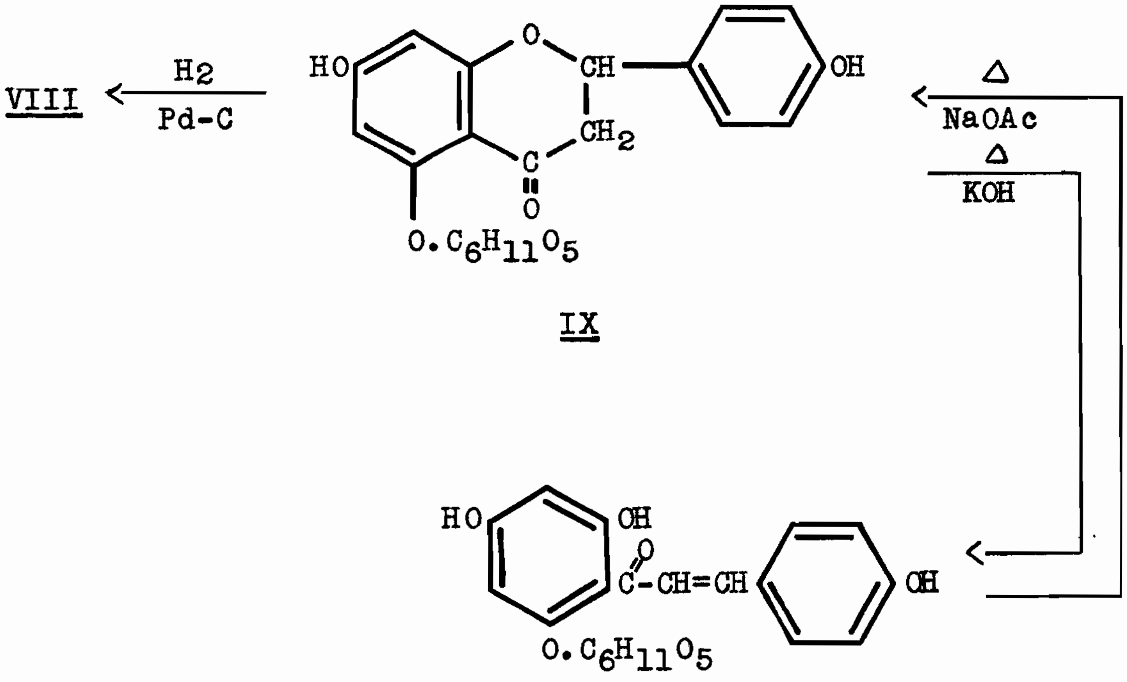
VII

In the bark of apple and pear (19), cherry, plum, and other rosaceous trees, has been found a chalcone-type glucoside, phlorizin (VIII). It was of the β-configuration, and hydrolyzable by emulsin.



VIII

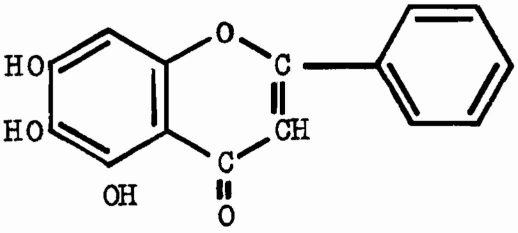
Representatives of the chalcones and flavones were closely related, and existed together in the bark of willow (Salix purpurea L.). Charaux and Rabate (20) obtained salipurposide (IX) and isosalipurposide (X) in the alcohol extract of the bark. Purification was effected by recrystallization from water. A 0.1% yield of the former was obtained by Zemplen, who showed the relationship between the two compounds, as well as the conversion of salipurposide to phlorizin, by the reactions connecting structures VIII, IX and X (21).



IX

X

Three hydroxyflavones from the bark of Oroxylum indicum Vent. have been identified (22). The alcohol extract was diluted with eight times its weight of water, and the resulting precipitate extracted with benzene. Crystals obtained from the ether extract of the aqueous filtrate were combined with those obtained from the benzene solution (total yield = 2.8%). The crystals were taken up in warm alcohol, treated with concentrated aqueous lead acetate, and the precipitate, after filtration, decomposed with hydrogen sulphide. This treatment gave baicalein (XI) in 0.5% yield. The filtrate was freed of lead ion with hydrogen sulphide and fractional crystallization led to 6-methylbaicalein (XII) and chrysin (XIII). The former could not be freed completely of chrysin.



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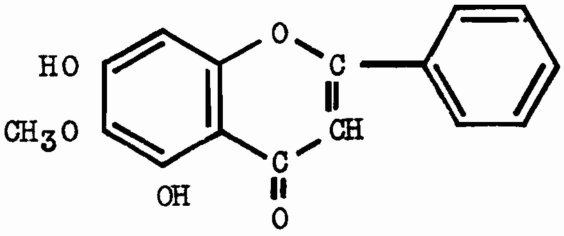
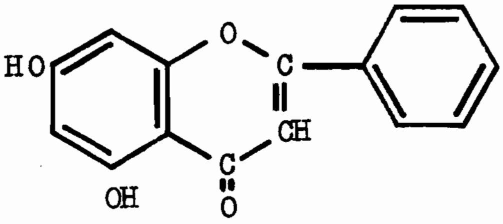
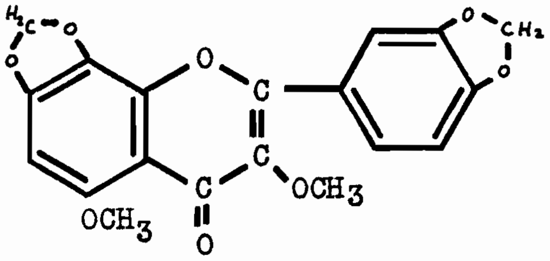


XII

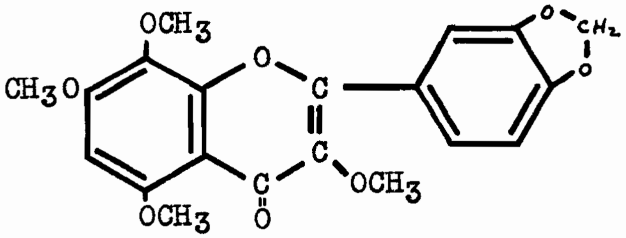


XIII

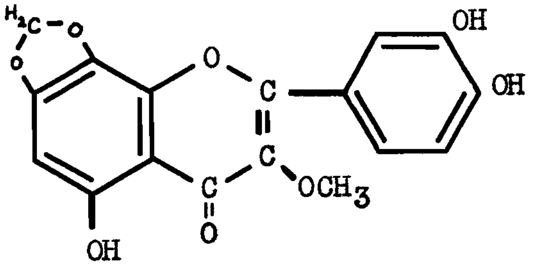
Briggs (23) has identified three flavonols, and proposed a structure of a fourth, from Melicope ternata bark. Extraction with trichloroethylene of the material removed from the bark by acetone, followed by the re-extraction of this extract with concentrated hydrochloric acid, was the basis of the separation. Precipitation of the acid solution with water and fractional crystallization yielded meliternatin (XIV) and meliternin (XV). Re-extraction of the trichloroethylene solution with sodium carbonate and sodium hydroxide gave wharangin (XVI, tentative structure) and ternatin (XVII), respectively.



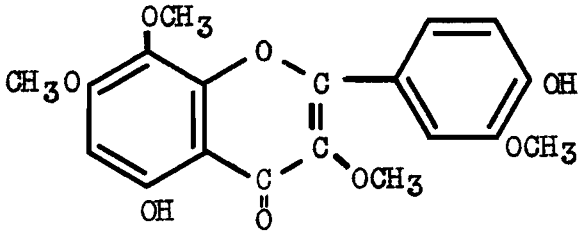
XIV



XV

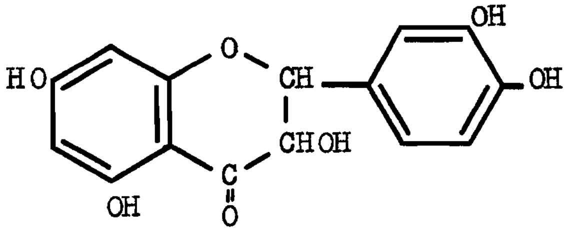


XVI



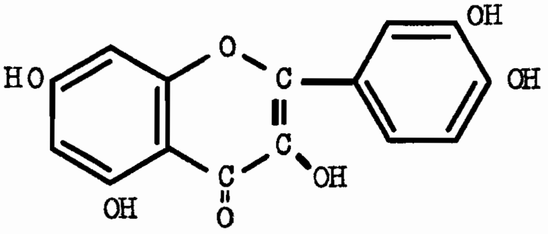
XVII

Ether extraction of benzene-extracted Douglas fir bark (24) recovered dihydroquercetin (XVIII), which on oxidation with 10% sulphuric acid in air yielded quercetin (XIX).



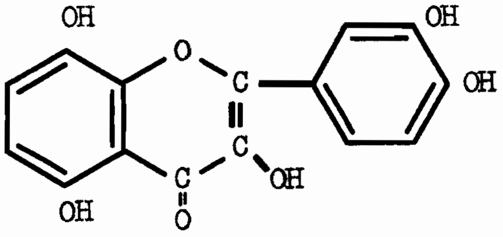
XVIII

The former was adsorbed by hide powder in the conventional tannin determination procedure (9), and was also found to combine with sheepskin skivers to give a white leather.



XIX

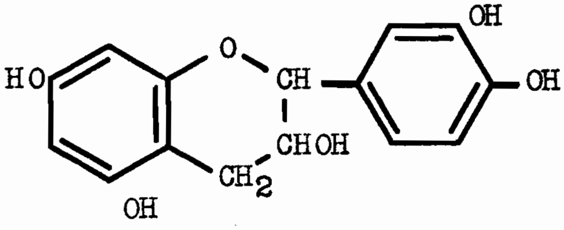
Flavonols in the barks of Ponderosa pine and Sugar pine have been removed by ether in 1.4% to 6.4% yields (25), the compound from the former bark having been determined to be 3,3', 4', 5,8-pentahydroxyflavone (XX), (26).



XX

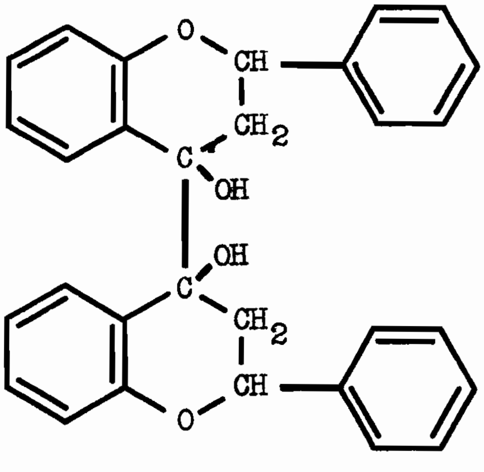
Tannins have been reviewed by Bishop (5), Russell (27) and Flinn (28), but the similarity of one class of tannins to the natural coloring pigments found in bark was worthy of brief recapitulation. Phlobaphene-producing tannins (Perkin classification (29)), or condensed tannins (Freudenberg classification (30)), have comprised the bulk of the natural tannins. The similarity of the decomposition products of the phlobatannins to those of the natural coloring matters has long been established. On acid hydrolysis, they yielded dark, insoluble, amorphous materials, called phlobaphenes.

While catechin (XXI) was not a tannin itself, it



XXI

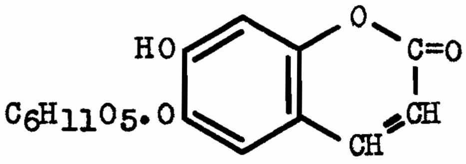
resembled phlobatannins in that it gave amorphous, water-insoluble, alcohol-soluble phlobaphene-like material on acid hydrolysis, also green colorations with ferric salts, and its fission products corresponded to those given by many phlobatannins (29). Freudenberg (30) expressed the view that each phlobatannin was a polymer of polyhydroxy derivatives of flavanol (V). However, the failure positively to identify phlobatannins as derivatives of this basic unit led Russell (31) to postulate a flavpinacol basis (XXII) for this class of tannins.



XXII

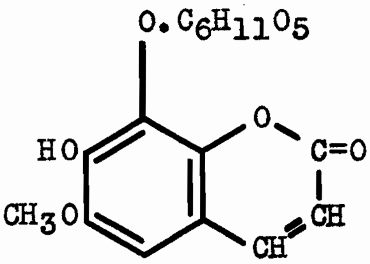
Thus phlobatannins were to be considered as phenolic hydroxyl derivatives of flavpinacol. Qualitative and some quantitative measurements on synthetic materials appeared to substantiate this postulate (32, 33).

Esculin (XXIII) was found by Zellner (34) in the bark of horse-chestnut (Pavia rubra, Lam.), together with tannins. The method of isolation consisted of taking up the concentrated ethanol-soluble fraction in water and allowing the insoluble phlobaphenes to settle. Treatment of the aqueous filtrate with neutral lead acetate solution precipitated the tannins, which were removed by filtration. The glucoside was then precipitated with basic lead acetate solution, the complex decomposed with hydrogen sulphide, and the product crystallized from aqueous alcohol. Esculin was the glucoside of 6,7-dihydroxycoumarin.



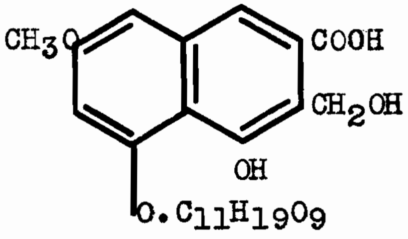
XXIII

Fraxin (XXIV) was a water-soluble glucoside in ash bark (35), which Zellner isolated (36) in the manner just described.

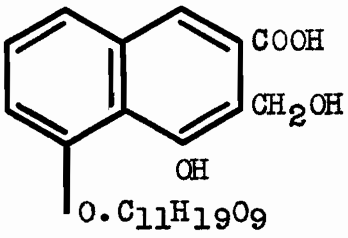


XXIV

The bark of Rhamnus japonica has yielded two glycosides of naphthoic acid, called α-sorinin (37) and β-sorinin (38), which were the lactones of structures XXV and XXVI, respectively. Hot 90-95% aqueous alcohol effected the extraction of these compounds from the bark. Concentration, extraction with absolute ethanol, followed by fractional recrystallization first from warm water and finally from 50% aqueous methanol (in the dark) completed the separation. The carbohydrate portions of both compounds were based on the disaccharide primverose (6-D-xylosido-D-glucose).

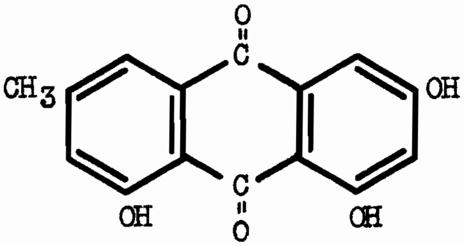


XXV



XXVI

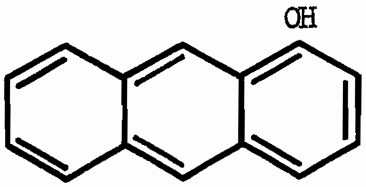
Bridel and Charaux (39) extracted buckthorn bark with cold water, and crystallization of the substance in the extract could be accomplished from 60% aqueous acetic acid, 33% aqueous pyridine, or amyl alcohol. Ethanol, methanol, or water gave unsatisfactory results, the solubilities in the first two solvents being too low, and in the latter, too high. An enzyme, which hydrolyzed the glycosides in a few hours, also went into aqueous solution, resulting in the precipitation of the aglycones (hydroxymethylanthraquinones). The bark was found to contain three water-soluble glucosides, the non-carbohydrate portion of which consisted of emodol (XXVII). The



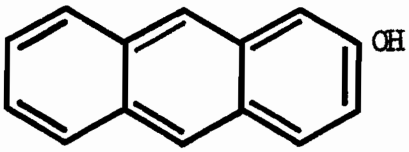
XXVII

first glucoside, present in small quantity, yielded glucose and emodol on hydrolysis with the enzyme diastase, while the second, present in greatest amount, gave glucose and franguloside, $C_{21}H_{20}O_9$(the rhamnoside of emodol), under similar treatment. The third was not affected by enzymes, but on acid hydrolysis yielded emodol and a reducing sugar.

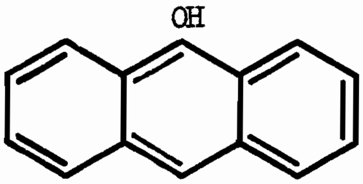
Continuing their investigation of buckthorn bark, Bridel and Charaux (40) extracted approximately 25% of the bark with water. From the water-extracted residue, ether removed frangularoside ($C_{21}H_{24}O_9$) in 3.5% yield. This compound appeared to have been formed during the water treatment by the enzymic hydrolysis of a more complex heteroside. Frangularoside, on hydrolysis, gave rhamnose and frangularol ($C_{15}H_{14}O_4$), which had an anthranol nucleus (XXVIII (a), (b), or (c)). The more complex heteroside was obtained in 10-12% yield from acetone-extracted bark by using hot ethanol, but could not be crystallized. Water-soluble, on hydrolysis with acid it yielded glucose and rhamnose in a molar ratio of 2:1, much emodol, and a small amount of a chloroform-soluble anthraquinone derivative, probably $C_{17}H_{12}O_4$.



XXVIII (a)



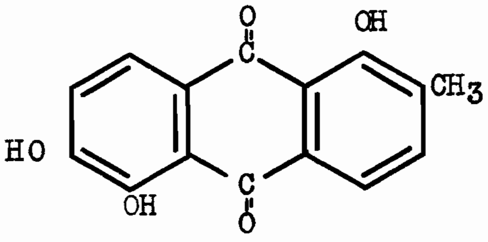
XXVIII (b)



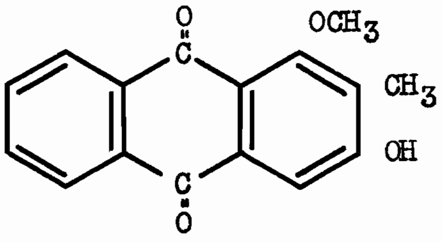
XXVIII (c)

Black alder bark contained glycosides similar to those found in buckthorn bark. The parent substance of franguloside was isolated in 10% yield, by alcohol extraction, as an amorphous, unstable powder (41). Heating the product in sulphuric acid resulted in a precipitate (40.3%), containing 59.73% of reducing sugars. Emodol was the chief non-glycosidic constituent. Rhamnodiastase gave a precipitate (26.52%), - chiefly franguloside and a little emodol, - containing 21.79% of reducing sugar, and an anthraquinone, $C_{17}H_{12}O_4$; treatment with yeast liberated franguloside and dextrose (molar ratio 1:2).

Three different methods of separation have been used to isolate glycosides and related compounds from the bark of the Coprosma genus (42). In the first method, the alcohol extract of Coprosma Australis, when cooled, yielded crystalline morindin, the rhamnoglucoside of morindone (6.8%). Evaporation of the alcoholic filtrate and treatment with excess barium or calcium hydroxides resulted in a soluble and an insoluble salt. Acidification of the former gave morindone (XXIX), of the latter, rubiadin-1 methyl ether (XXX), each in 5.1% yield. Morindin could also be extracted by water, and a subsequent extraction with acetone removed the two other constituents. The third method, chromatographic separation of the alcohol extract on magnesium oxide, was also successful.



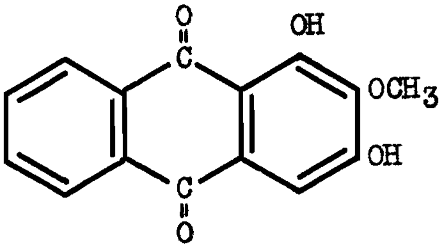
XXIX



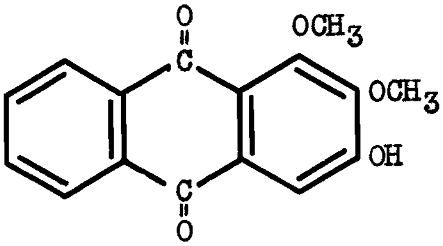
XXX

Coprosma acerosa was found to contain glycosides, as well as the aglycones in the non-glycosidic state (43). The method of isolation used was similar to the foregoing, with the exception that all glycosides were hydrolyzed with acid, and the aglycones chromatographed on magnesium oxide. Anthragallol-2 methyl ether (XXXI), anthragallol-1,2 dimethyl ether (XXXII), 1,6-dihydroxy-2-methylanthraquinone (XXXIII), rubiadin (XXXIV), and rubiadin-1 methyl ether (XXX) constituted the products.

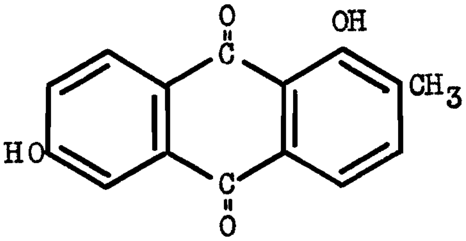
Anthraglycosides were also present in the barks of



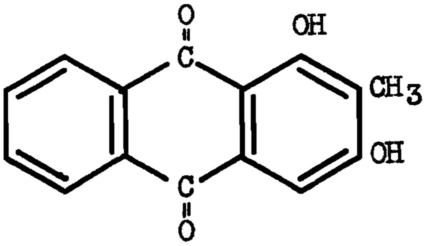
XXXI



XXXII



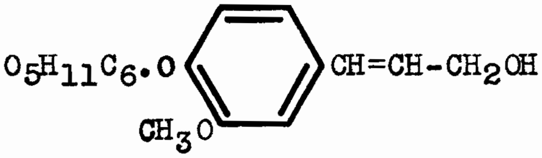
XXXIII



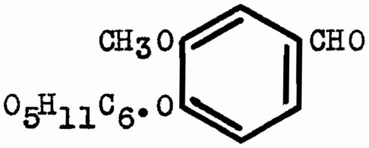
XXXIV

various types of Italian Rhamnaceae, in yields of from 1% to 4% (44).

Coniferin (XXXV), found in coniferae barks and the cambium layer of Douglas fir (45), was readily converted to vanillin glucoside (XXXVI) by chromic acid oxidation. The latter has been found in crystalline form on the smooth areas of beech bark, which have been exposed to sunlight. The only purification necessary was recrystallization from ethanol containing a little water, (17).

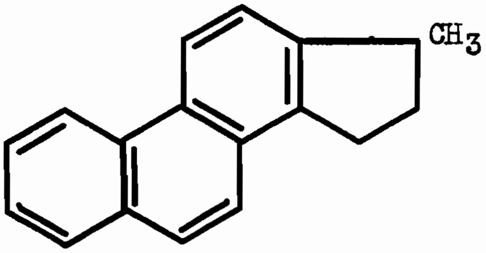


XXXV

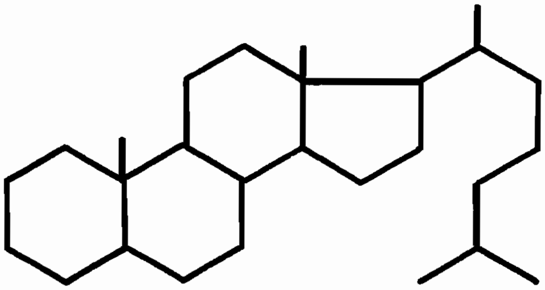


XXXVI

Saponins (59) were complex glycosides capable of giving solutions which foam on shaking. The aglycones were of two main types, the steroid sapogenins and the triterpenoid sapogenins. The former, on dehydrogenation with selenium, gave methyl-cyclopentenophenanthrene (XXXVII), a product obtained from sterols and allied substances by similar treatment, and thus the carbon skeleton of this type was represented by structure XXXVIII; the latter type, on dehydrogenation with

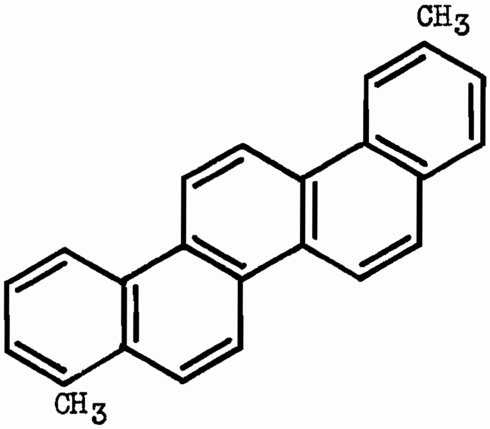


XXXVII

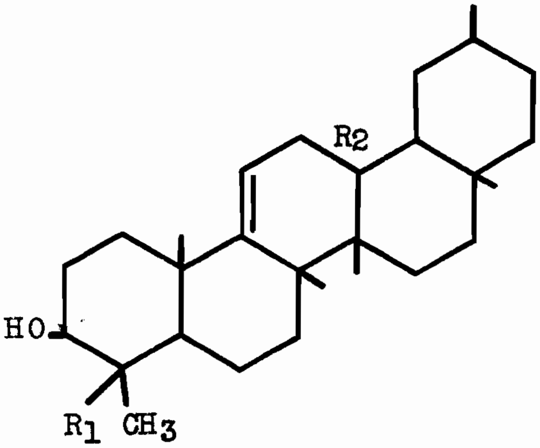


XXXVIII

selenium, gave 1,2,7-trimethylnaphthalene, a hydrocarbon which has also been obtained from various triterpene compounds, and also 1,2,3,4-tetramethylbenzene, 2,7-dimethylnaphthalene, 1,2,5,6-tetramethylnaphthalene, and 1,8-dimethylpicene (XXXIX), from all of which data, structure XL was postulated as being fundamental. The aqueous extract of American larch bark gave



XXXIX



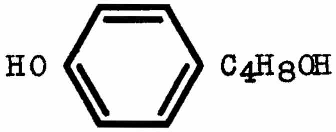
XL

where R_1 = CH_2OH, CH_3, CHO, CH_3 and CH_3,

R_2 = COOH, COOH, COOH, CH_2OH, CH_3, respectively, in various compounds.

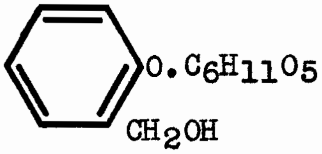
voluminous and persistent froth when shaken, an observation indicative of saponins, but no crystalline glucosides were isolated (46). Addition of saturated ammonium sulphate or neutral lead acetate to the aqueous extract of Fagara bark precipitated a saponin (47). Guaiac bark contained a saponin ($C_{30}H_{47}O_3 \cdot C_6H_{11}O_5$), the aglycone of which was a dextro-rotatory monohydroxymonocarboxylic acid (48). The methanol extract of the bark of Kalopanax ricinifolius, Miq. on treatment with lead acetate gave a precipitate. Decomposition of the precipitate and purification from alcohol and acetone also yielded a saponin (49).

Betuloside ($C_{16}H_{24}O_7$), a glucoside from white birch, was of the β-configuration, and was hydrolyzed by emulsin (50). Its aglucone was represented by XLI (51).



XLI

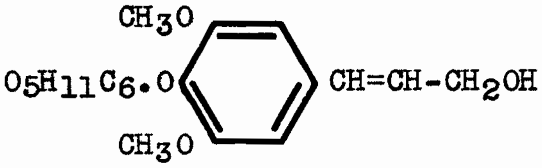
Salicin (XLII), the β-glucoside from willow bark, was present in yields of up to 3% (52), and has also been found in British Columbian willows and poplars (53). Populin, the 6-benzoyl derivative of salicin, occurred in poplar barks (53) and was not hydrolyzed by emulsin.



XLII

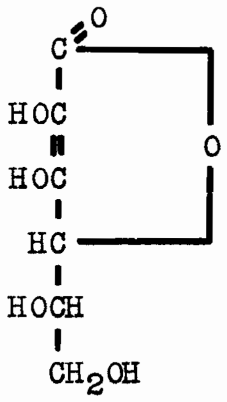
The bark of the Bignoniaceae contained catalposide (54), which was isolated by extraction with alcohol, concentration, re-solution in water, precipitation with neutral lead acetate, and decomposition of the lead precipitate with sulphuric acid. Crystallization was accomplished from water. The aglucone was unstable, and darkened as soon as it was isolated. The structure has not yet been elucidated (55).

Treatment similar to the foregoing yielded syringoside (XLIII) from the bark of Scrophulariaceae (54).

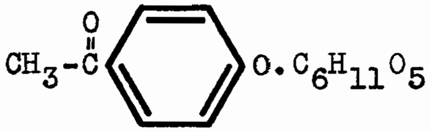


XLIII

A glucoside of vitamin C (XLIV) or its ester, has been isolated from both the root and trunk bark of Celastracea (56), more being found in the former than in the latter. Bridel (57) and Jowett (58) have described the finding of piceoside (XLV) in black willow bark. Methods of isolation were similar to those which have already been described using



XLIV



XLV

lead acetate. Piceoside was of the β-configuration and hydrolyzable by emulsin.

DISCUSSION OF RESULTS

The complex mixture of non-dialysable substances originally obtained by Bishop (5) had resulted from a series of physical separations. When all possible methods of fractionation, such as partial solubility in a solvent, precipitation, dialysis, etc., had been exhausted, only chemical means remained for furthering the investigation. However, important changes had occurred in fraction 21 during the first six months of storage. The copper-reducing power of the mixture increased from zero to 27.4%, while the solubility in water fell from 100% to 24%. At room temperature and pH 6, acid hydrolysis seemed unlikely, if not impossible, and the cleavage of sugars was attributed to enzyme action, the mildest of all degradations. This enzyme probably originated in the cold water extract of the bark, which had been eventually combined with other extracts prior to dialysis.

Confirmation of Previous Analyses

A determination of the extent of these changes during a further 12 months of storage was clearly indicated as the first step in the investigation of the components of the non-dialysable fraction. Bishop had reported the amount of tannin present as 52.5%, a value which could be reduced to 39.0% by treatment with emulsin. The present tannin content of 36.3% was in excellent agreement with the latter figure. The quality of the tannin was apparently the same, since a sample caused a rise in the shrinkage temperature of rawhide of 17°, as compared to a previous increase of 15°.

Bishop also reported that treatment with emulsin increased the copper-reducing power from 27.4% to 37.0%, whereas acid hydrolysis resulted in an increase to 54.0% as glucose. The inability of emulsin to cleave 17.0% (i.e. 54.0 - 37.0) of the combined sugar suggested either polysaccharides or glycosides of other than the β-configuration. The present increase caused by emulsin from 20.2% initial free copper-reducing power as glucose to only 22.0% indicated that the enzymatic hydrolysis during 18 months' storage was substantially complete. Acid hydrolysis now increased the sugar value to 36.6%. The 14.6% (i.e. 36.6 - 22.0) of combined sugar remaining was not far from the previous figure of 17.0%.

Preliminary Investigations

Detailed sugar analyses before and after acid hydrolysis, fermentation, and treatment with emulsin, are given in Figs. V and VI, and require little explanation. The presence of approximately 8.6% of non-fermentable copper-reducing power, in contrast to only 1.5% after acid hydrolysis, was attributed to non-carbohydrate material. Fermentation with yeast permitted the retention of such material in solution, whereas acid hydrolysis converted it to dark-brown, insoluble substances, which were always removed by filtration, prior to the determination of the copper-reducing power. The extraction of copper-reducing material from aqueous solution by butanol confirmed Bishop's re-

FIGURE V

Sugar Analysis of Fraction 21

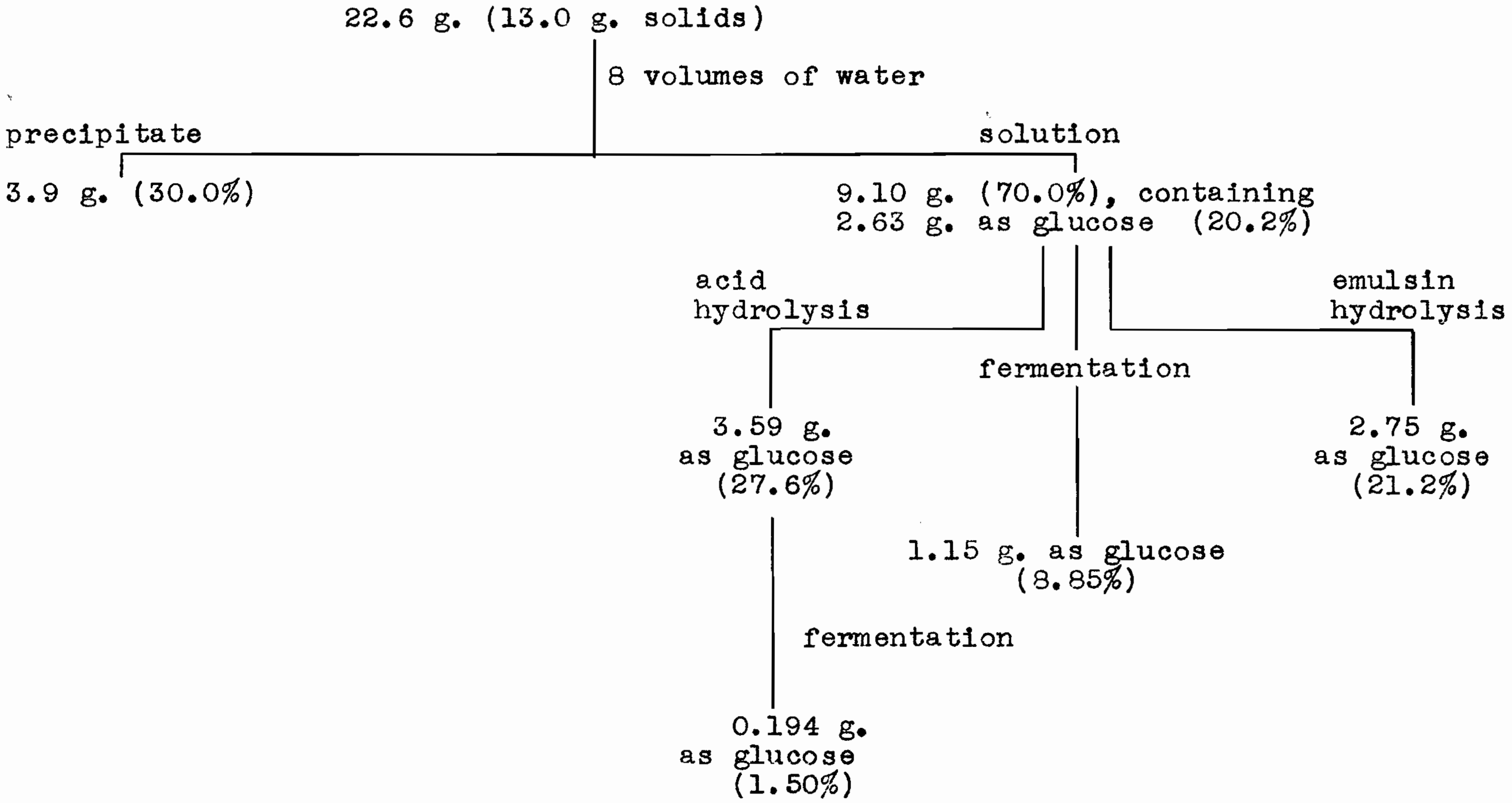
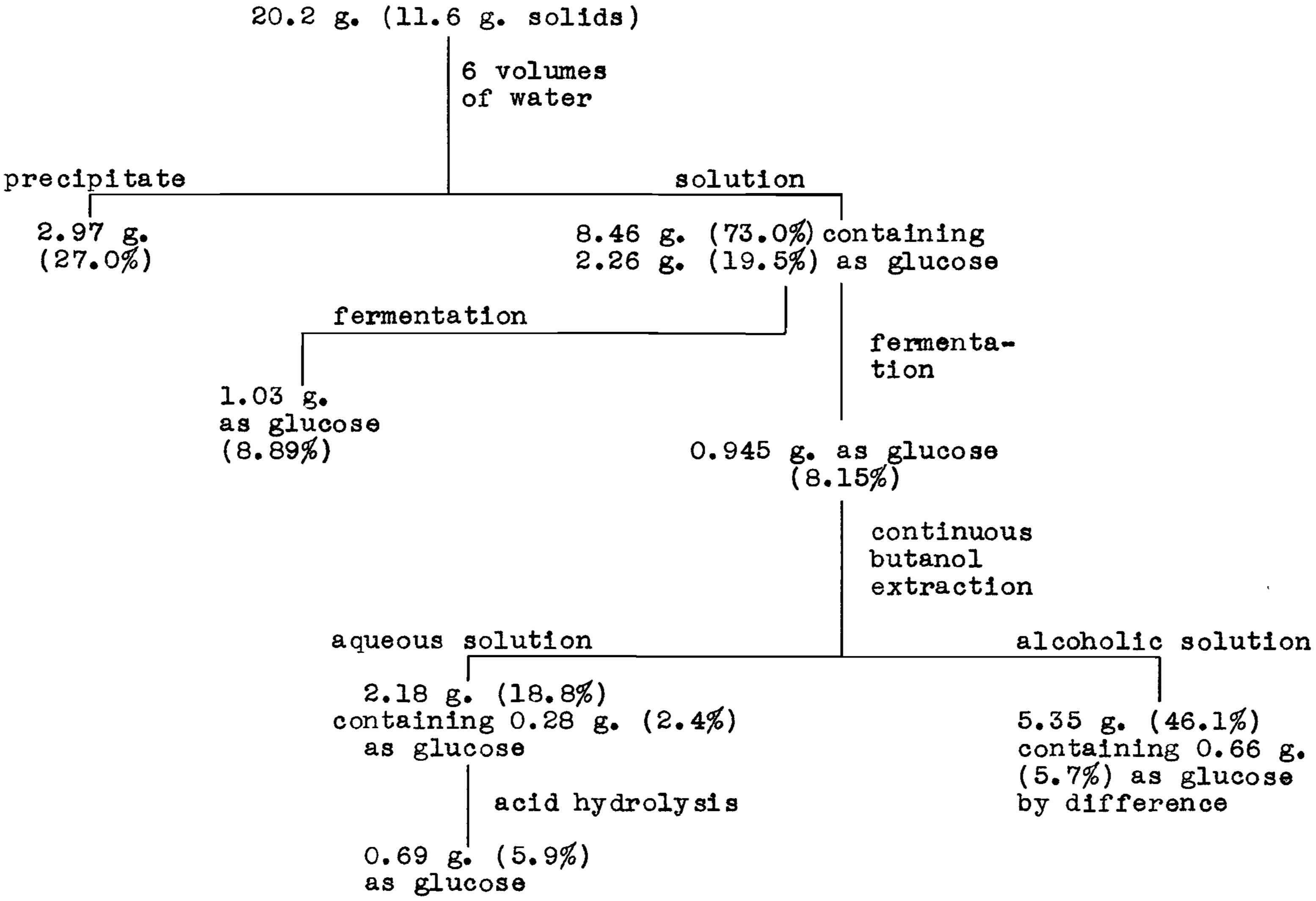


FIGURE VI

Sugar Analysis of Fraction 21



port of a similar occurrence.

The thick, oily fraction 21 was miscible with alcohol and "basic" organic solvents; dioxane and morpholine were partially miscible. No water-immiscible liquids having any effect were found, which excluded fractionation by extraction of the aqueous mixture (Table III). Dilution with water precipitated a dark-brown gum, varying in amount from 23% to 30% of the total solids, as the amount of water added was increased from 2 volumes to 8 volumes. This result was in almost direct contrast to the observations made by Bishop (i.e. 76% precipitated by water). Communication with Dr. Bishop confirmed his figure, and provided

TABLE III

EFFECT OF VARIOUS LIQUIDS ON FRACTION 21

Solvent	Observations
Hydrocarbon solvents	No effect
Alcohol	Almost complete miscibility
Water	Partial miscibility and a black gummy precipitate
Acetone	Partial miscibility and a black gummy precipitate
Pyridine	Complete miscibility
Dioxane	Almost complete miscibility
Morpholine	Partial miscibility and a black hard precipitate
Aqueous acid	Varying degrees of precipitation and partial miscibility
Aqueous alkali	Complete miscibility

the information that the copper-reducing power of the diluted aqueous mixture increased on standing. An enzyme, which split the polyglucosides in the seeds of Adenium multiflorum to monoglucosides, was isolated by Hunger and Reichstein, and actually used for this purpose (89). It was the author's suggestion that enzymatic hydrolysis of the presumed polyglucosides to monoglucosides was also accompanied by an enzymatic cleavage, or other change, of the aglycones. Assuming that the larger, more complex aglycones, on liberation, were water-insoluble, further cleavage could produce smaller, water-soluble compounds. This idea fitted in with Freudenberg's (30) class of hydrolyzable tannins (split by acids or hydrolyzing enzymes), but conflicted with other observations, which indicated the presence of materials similar to phlobatannins. The latter belonged to the condensed tannin class, which were not cleaved by acids or enzymes. No further investigation of the water-insoluble material was carried out.

The precipitation of a dark-brown gum from fraction 21 by dilute mineral acids pointed to acidic components, which were tentatively considered to originate in phenolic hydroxyl groups because the solubility in dilute aqueous sodium bicarbonate was only partial, although that in dilute aqueous sodium hydroxide was complete. Carboxylic acids would probably have been completely soluble in both these solutions. Alcohol-insoluble basic salts could be formed by the addition of alcoholic potassium hydroxide, or amines, to alcoholic solutions of the mixture.

The former salts were only soluble in water, while the latter were soluble in pyridine as well. Since the mother liquor from the amine precipitation was dark-brown, a possible means of fractionation was suggested, but no reproducible results were achieved using this line of investigation. Both this alcoholic mother liquor and also alcoholic solutions of fraction 21 showed fluorescence when irradiated with ultra-violet light.

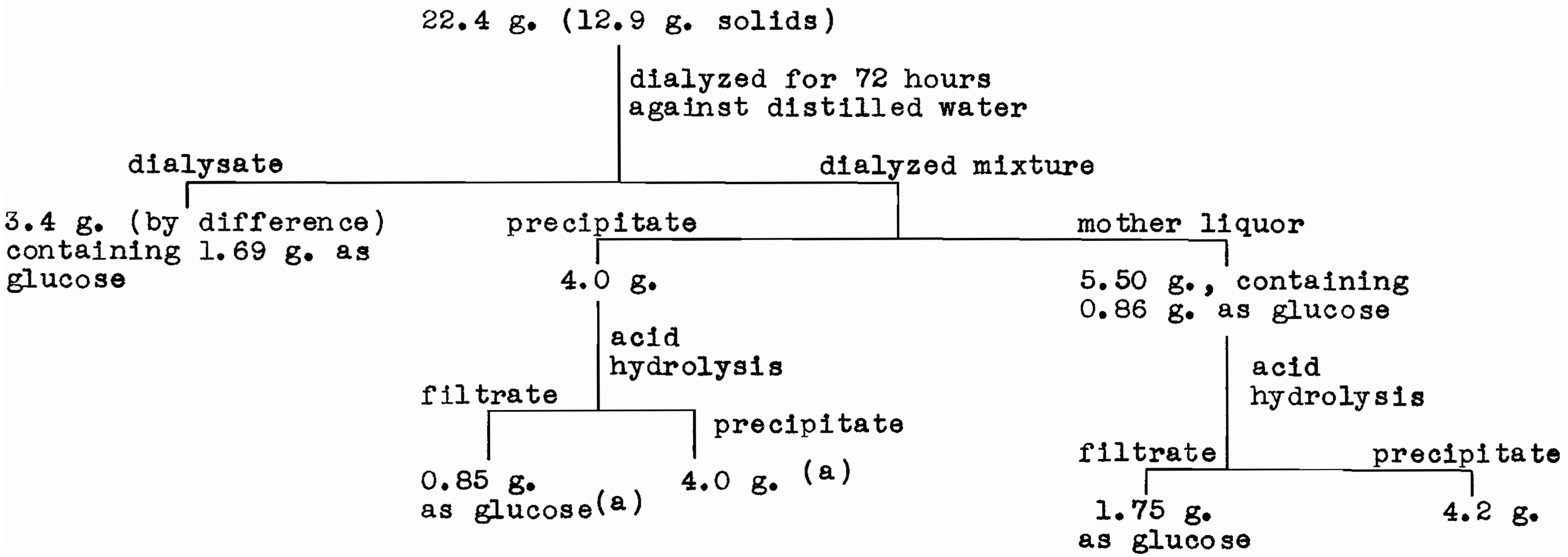
To obtain a sugar-free mixture, dialysis was carried out (Fig. VII). The presence of 26.4% of the total solids in the dialysate, whereas the total sugar content was only 20.2% as glucose, suggested that material other than sugar was passing through the membrane. Thus dialysable substances had been liberated (presumably by enzymes) from the originally non-dialysable fraction, and complete dialysis of the mixture was decided on as the initial step in the investigation.

Fractionation of Fraction 21

In order to minimize sampling errors resulting from operations carried out at different times, a large-scale dilution of fraction 21 with 2.5 volumes of water was carried out, resulting in the separation of 4,600 g. of solids into 1,200 g. of water-insolubles and 3,400 g. of water-solubles. A 435-g. sample of the latter was subjected to dialysis in a continuous vacuum dialyzing apparatus. In the course of this operation, 94.4 g. of gum was precipitated by the dilution with water of the aqueous solution in the sacs, and was taken up in alcohol. After 50 days the dialysis was stopped. At this point 277.7 g.

FIGURE VII

Preliminary Dialysis of Fraction 21



(a) The apparent yield of 4.85 g. was 121% of the 4.0 g. hydrolyzed, but the discrepancy could not be explained.

had appeared in the dialysate, while 63 g. remained in the aqueous residue. The presence of dialysables amounting to 49.5% of this 63-g. residue was shown by further dialyzing an aliquot of the aqueous solution for one month and it seemed probable that all the residue would have passed through the cellophane membrane eventually. Dialysis data are summarized in Table IV. Extraction with butanol removed 70% of the solids in the aqueous solution remaining after 50 days' dialysis, but neither solution was investigated further, partly because Bishop (5) had been active in a similar field.

The dialyzable portion exhibited an important change in property from the original fraction 21, in that it could be dried thoroughly, but still exhibited complete and ready solubility in water. Although this property of re-solution after drying was not generally characteristic of tannins, the adsorption of over 50% of the fermented, dialysable material on hide powder clearly showed that tanning agents were present. As has been mentioned, fraction 21, as received, was not completely water-soluble, but 25% aqueous methanol gave complete solution. The passage of such a solution through a resin column of Amberlite IRA-400 removed varying portions of material, but in all cases the filtrates could be completely dried and the residues taken up in water again. While these experiments could not be repeated quantitatively, it appeared that adsorption on the resin approximated the results of the much more tedious dialysis. Dialysis was nevertheless retained, because it transpired

TABLE IV

DIALYSIS OF FRACTION 21

Weight of solids dialyzed, 435 g. "Glucose" content, 123 g.

Dialysis Run	Time Intervals (days)	Solids Dialyzed (g.)	"Glucose" Dialyzed (g.)	Solids after Fermentation (g.)	"Glucose" after Fermentation (g.)	"Glucose" after Acid Hydrolysis (g.)	Precipitate(a) from Acid Hydrolysis (g.)	"Glucose" after Fermentation of Acid Hydrolysate (g.)	Material adhering to Sacs(b) (g.)
a	3	86.6	45.1	45.8	7.8	13.7	12.2	1.74	...
b	2	34.2	12.9	22.2	2.6	7.1	7.6	1.08	...
c	2	28.2	7.6	23.4	2.4	7.7	10.8	1.15	...
d	3	12.6	2.6	9.9	1.1	3.9	3.8	0.68	66.0
e	4	18.9	3.0	17.2	2.0	6.6	6.6	0.94	...
f	4	28.0	4.3	26.6	3.3	10.1	12.4	1.15	...
g	5	19.0	2.5	18.7	2.2	7.6	9.0	1.06	21.3
h	5	17.8	2.4	15.5(c)	2.1(c)	5.4	6.9	0.89	7.1
i	4	8.2	1.0	8.1	0.9	2.9	4.1	0.43	negligible
j	13	15.9	2.5	15.8	2.4	5.0	7.5	0.95	...
k	15	8.3	1.1	8.2	1.1	2.6	3.5	0.22	negligible
Totals	50	277.7	85.0	211.4	27.9	72.6	84.4	10.29	94.4

(a) Weights of precipitates calculated from data determined on aliquots.
(b) Sacs changed after dialysis runs d, g, h, i.
(c) Figures are low because of slight loss during centrifuging.

that purification by chromatographic means was even more effective at a later stage. The remaining 277.7 g., 85.0 g. as glucose, of the dialysable substances was completely fermented and taken to dryness, where it appeared as a dark-brown, amorphous resin.

The Fermented, Dialysable Fraction

The 13.2% of copper-reducing power (as glucose), not eliminated by fermentation, could be increased to 34.4% by acid hydrolysis, and the latter figure again reduced to 4.86% by fermentation. The fraction thus contained 29.5% (i.e. 34.4 - 4.86) of glucose combined in a non-fermentable form. The residual 4.86% was attributed to water-soluble, non-carbohydrate copper-reducing material, and the 8.3% (i.e. 13.2 - 4.86) difference of non-fermentable substance before and after acid hydrolysis was assumed to be caused by the loss of copper-reducing material as water-insoluble precipitates. These precipitates were presumably of a non-carbohydrate nature, since they would have been hydrolyzed to soluble, reducing sugars if the reverse was the case. All percentages were calculated from data in Table IV.

The absence of galactose in the fermented, dialysable fraction did not prove the absence of galactosides in the original mixture, since the yeast used would ferment galactose. However, Bishop reported no galactose in any of his fractions from white spruce bark. The present finding of 3.4% of "furfural" would account for slightly over half of the non-fermentable copper-reducing power as pentose (6.8%). The method of analysis, however, did not differentiate between furfural, methyl furfural and hydroxymethylfurfural, and the recovery of small percentages of one or other of these compounds did not necessarily imply the presence of pentose, pentosans, or pentosides.

The solubilities of the fermented, dialysable fraction closely paralleled those of the original mixture (Table III). Hydroxylated and "basic" organic solvents, and water gave complete, or very nearly complete, solution, while hydrocarbon solvents had no effect. Alcohol-insoluble basic salts could still be formed, and probably also acid salts, such as chlorides and picrates, but the existence of the latter was not proven. Fractional precipitation of these salts did not appear to achieve any worth-while results. Chromatographic adsorption with various adsorbents failed to effect any separation. A possible lead in the investigation originated in the use of mixed solvents. Whereas ether, diethyl ketone, and a mixture of amyl alcohol-acetophenone, had little or no extractive powers, the addition of picric acid to each of these liquids rendered them capable of extracting at least a portion of the material, particularly in the case of diethyl ketone. This line of investigation was only touched on, because of the prolonged delay in procuring the relevant article by Schudel (76), and other methods were finally chosen in its place. Nevertheless, the possibilities in this means of separation were not to be dismissed completely.

Many attempts were then made to devise methods of separation based upon the formation of derivatives methylated with diazomethane or with dimethyl sulphate and alkali; also upon acetates made by the pyridine - acetic anhydride method. Although these attempts failed in their main purpose, they led to the recognition that the amorphous, brown acetates were soluble in a wide range of organic liquids, ether and petroleum ether excluded. This property was of especial value for chromatographic methods of separation, for the acetates were held in solution by solvents less tenaciously than the original substances, which permitted their adsorption, and a wider range of liquid mixtures was available for their successive elution. Small-scale chromatographic experiments on alumina, using organic solvents of gradually increasing eluting power, separated the crude acetate mixture into fractions of approximately 50%, 30% and 20%. It was also noted that if tannins were removed by hide powder from the sample prior to acetylation, the chromatographic separations were, in general, more successful. This fact, in combination with the decision that tannins were not of especial interest in this investigation, led to the following plan of separation: adsorption of the tannins on excess hide powder, acetylation of the non-adsorbed material, chromatographic separation of the acetate mixture, and subsequent examination of the fractions obtained.

Two separate preparations were used for the large-scale acetylations. The first employed the fermented, dialysable fraction, which was prepared as already described, followed by treatment with newly-purchased standard hide powder.

The non-adsorbed material (39.8%) was then acetylated with pyridine and acetic anhydride in 62.5% yield by weight. This product was used in chromatographic Run I (Table VII). An unsuccessful attempt was made to recover the 37.5% of product remaining in the aqueous filtrate, by reacetylating the dried residue. No water-insoluble material was formed, and the common solubilities of the organic matter lost and the by-products prohibited any direct separation. The sequence of operations in the second preparation was changed in order to shorten the long period of dialysis (50 days) formerly employed. A portion of the water-soluble part of the original fraction 21, after dilution with 2.5 volumes of water, was completely fermented and filtered, and the filtrate, containing 74.3% of the total solids, was treated with an excess of newly-purchased hide powder. The recovered non-adsorbed material amounted to 40.4% of the solids so treated. Having thus removed 70.0% of the original solids, a dialysis of only 3 days against distilled water enabled 35% to pass through the cellophane membrane. This amount was judged to be sufficient for the purpose, and the operation was discontinued while still incomplete. Acetylation of the dialysable fraction with acetic anhydride and pyridine was accomplished in only 43.8% yield, but no explanation of this lowered yield could be offered. This acetate mixture was used in chromatographic Run II (Table VIII).

The results of the separations by chromatography were summarized in Table V. A precaution which had to be stressed was that alumina more active than Brockmann grade III was not to be used. A 23.0-g. sample of crude acetate was irretrievably adsorbed on alumina of grade I, and had to be discarded with the adsorbent. While the alumina used in Runs I and II was grade III, that used in the former was slightly less active than that used in the latter.

Fraction I - C, essentially methanol-insoluble but water-soluble, was optically inactive, and its high ash content discouraged further investigation. The minor fractions I - C and II - B were similar. Fraction II - D, insoluble in methanol and water, appeared inorganic in nature, and was discarded without further analysis.

Fractions I - D and II - C were inadvertently mixed during the initial stages of their investigations and will have to be discussed together. Briefly, a thorough search for a means of purifying this crystalline material resulted in failure. Recrystallization could not be carried out because of the lack of a suitable solvent; precipitation from methanol solutions with non-solvents led to amorphous, colored solids; deacetylation with sodium methoxide was invalidated by inability to separate the inorganic salt from the reaction mixture; chromatographic adsorption and recovery gave no separation. In the main experiment, 7.2 g. out of 30 g. of the mixed acetates failed to dissolve in methanol, and fractional precipitation of the extract with benzene recovered the remainder as five fractions, with ash contents ranging from 19% to 51% (Table IX). Since in all likelihood, the methanol-insoluble solid, 7.2 g.,

TABLE V

RESULTS OF CHROMATOGRAPHIC SEPARATION OF THE ACETATES

Fraction	Eluted with	Weight (g.)	Yield (%)	Ash (%)	Physical state
Run I. Sample 29.0 g., acetyl 39.4%					
I - A	C_6H_6	13.8	47.6	0	amber syrup
I - B	5% MeOH in C_6H_6	9.6	33.1	0	amber syrup
I - C	MeOH	2.5	8.6	64.6	brown solid
I - D	5% HOAc in MeOH	10.0	34.6	ca. 45(a)	brown crystals
		Total	123.9		
Run II. Sample 36.2 g., acetyl 40.9%					
II - A	10% MeOH in C_6H_6	17.1	47.2	0	amber syrup
II - B	MeOH	1.1	3.0	...(b)	brown solid
II - C	5% HOAc in MeOH	27.9	77.2	ca. 45(a)	brown crystals
II - D	15% HOAc in MeOH	21.9	60.5	ca. 100	gelatinous solid
		Total	187.9		

(a) after admixture (b) similar to I - C was also largely inorganic, the greater-than-100% recovery from the columns was explained. Large amounts of some inorganic impurity, possibly sodium aluminates to judge from the ash alkalinities, had been removed together with the portions of the crude acetate mixtures most strongly adsorbed on the column. Further analysis of these fractions was abandoned.

Fractions I - A, I - B and II - A were converted to amorphous, light-brown powders by solution in methanol and reprecipitation with water. A portion of each was then reacetylated with pyridine and acetic anhydride, but failure to increase the acetyl contents substantially and almost quantitative recoveries showed that the original fractions were almost completely acetylated. Good checks were obtained for all carbon, hydrogen, acetyl, methoxyl, and molecular weights determined on the acetates, and the deacetylated and methylated products derived from them. The reacetylation and subsequent extractions with water made it probable that any traces of adsorbed methanol had been thoroughly removed from the acetates, which were free of ash and nitrogen, while prolonged extraction of the deacetylated and methylated products with pentane served to achieve the same end.

The results for fraction I - A corresponded to the molecular formula $C_{18.06}H_{18.46}O_{8.12}(C_2H_3O)_{6.11}(OCH_3)_{0.77}$ and the molecular weight 652. Although the carbon, hydrogen and acetyl analyses easily fitted the nearest formula with integral numbers, one mole of methoxyl in the observed molecular weight of 625 required OCH_3 4.95%, whereas only 3.66% was found. This discrepancy strongly suggested that the substance was not a chemical individual, but a mixture containing components of different methoxyl contents. The deacetylation of such a mixture would produce a 60.5% yield of a substance with the empirical formula $C_{18.06}H_{24.57}O_{8.12}(OCH_3)_{0.77}$ and of molecular weight 395. When, however, the deacetylation was carried out with excess barium methoxide, the product was isolated in only 27% yield by weight, and had analyses corresponding to $C_{16.93}H_{22.67}O_{7.7}(OCH_3)_{1.3}$. Although molecular weights were not observed, the decrease in copper-reducing power, before and after fermentation of the acid-hydrolyzed material, corresponded to one glucose residue in a molecular weight of 410, a value in good agreement with that expected. The results again suggested that the original acetate was a mixture, about 45% of which yielded a more highly methoxylated product on deacetylation, while the remainder was substantially methoxyl-free, and was lost. The well-known instability of β-phenolic glucosides toward alkali might have been of relevance in this connection, but the more likely hypothesis was that the missing portion had been retained by the Supercel used to clarify the solution after deacetylation. All that could be inferred about the missing half was that it probably possessed a molecular weight close to the calculated value of 395, because the half isolated probably had this value. The thorough methylation of the deacetylated product with diazomethane gave, in nearly quantitative yield, a product whose empirical formula, $C_{15.25}H_{18.64}O_{6.75}(OCH_3)_{2.25}$, indicated that one additional methoxyl group had been introduced into the molecule. This group was presumably of a phenolic rather than an acidic nature, since neither the original acetate nor the deacetylated material showed any acidic properties. It was at once obvious, however, that if formulae having 9 oxygen atoms were chosen for the deacetylated and for the methylated products, the carbon skeleton of the latter contained about 15 instead of about 17 carbon atoms. Alternative formulae, of only slightly greater molecular weight, could be obtained by keeping the carbon contents uniform at, for example, 18 atoms, but in this case, the oxygen content increased in an inexplicable way toward 10 or 11 atoms during methylation with diazomethane. Since the results with fractions I - B and II - A were closely similar in trend, although different in detail, it was finally concluded that no precise structural information was to be obtained by further elementary analyses. All the formulae given in Table VI, indeed, lay within the ranges C, 54.9 to 58.3; H, 5.95 to 7.0; acetyl, 38.7 to 40.4%, and in several cases alternative formulae fitted the analyses even when the molecular weight was approximately known by independent methods.

As might have been expected from their similar positions on the chromatographic column, fractions I - A and II - A tended to agree in elementary composition, and particularly in possessing 6 acetyl groups, whereas fraction I - B had 7. The methoxyl contents of all three acetates, however, were in good agreement, and so also were their specific rotations of $[\alpha]^{25°}_{D}$ - 19.6°, - 17.4° and - 22.0°, respectively, in methanol.

TABLE VI

ANALYTICAL DATA FOR FRACTIONS I - A, I - B AND II - A

		Moles of		Mol. Wt.	
Acetates	Formula	Acetyl	OCH_3	Calcd.	Obs.
Fraction I - A	$C_{31}H_{40}O_{15}$	6	0.77	653	625(a)
I - B	$C_{37}H_{46}O_{17}$	7	0.76	763	771(a)
II - A	$C_{32}H_{42}O_{15}$	6	0.74	667	687(a)
Deacetylated Products		Hydroxyl			
Fraction I - A	$C_{18}H_{26}O_9$	6 (?)	1.34	387	410(b)
I - B	$C_{17}H_{24}O_9$	7 (?)	1.36	372	505(b)
	$C_{19}H_{26}O_{10}$			415	
	$C_{21}H_{28}O_{11}$			457	
II - A	$C_{21}H_{30}O_{11}$	6 (?)	1.13	459	421(b)
	$C_{23}H_{34}O_{12}$			503	
Methylated Products (diazomethane)					
Fraction I - A	$C_{18}H_{26}O_9$	5 (?)	2.43	387	...
	$C_{19}H_{28}O_{10}$			417	
I - B	$C_{22}H_{32}O_{10}$	5 (?)	3.58	457	...
	$C_{24}H_{34}O_{11}$			499	
II - A	$C_{22}H_{32}O_{10}$	4-5 (?)	2.80	457	...
	$C_{24}H_{34}O_{11}$			499	

....

TABLE VI (cont'd)

ANALYTICAL DATA FOR FRACTIONS I - A, I - B AND II - A

		Moles of		Mol. Wt.	
Acetates	Formula	Acetyl	OCH_3	Calcd.	Obs.
Aglycones (calcd. from acetates)		Hydroxyl			
Fraction					
I - A	$C_{13}H_{18}O_4$	3 (?)	ca. 1	...	...
I - B	$C_{17}H_{22}O_5$	4 (?)	ca. 1	...	...
II - A	$C_{14}H_{20}O_4$	3 (?)	ca. 1	...	...

(a) By ebullioscopic method using methanol solutions.

(b) Weight equivalent to production of 1 mole of fermentable glucose after acid hydrolysis.

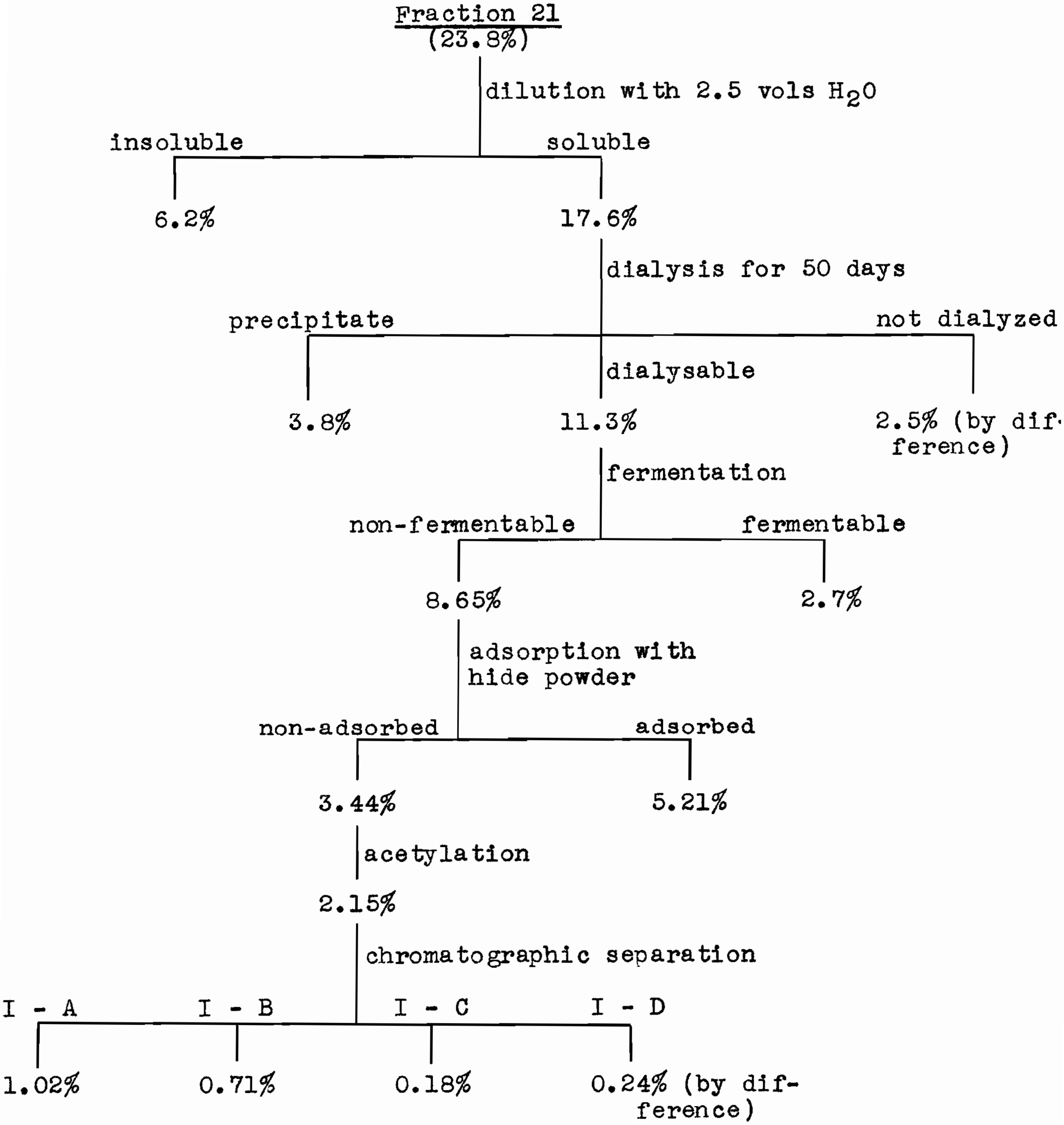
The deacetylations, which gave yields of only 45% to 59%, presumably converted the acetyl to an equal number of hydroxyl groups, and in each case the methoxyl substitution rose to a uniform but unexpectedly high value. Fraction I - B differed from the others in that the mole of fermentable sugar it was known to contain could not be liberated by the action of emulsin, and because diazomethane introduced two methoxyl groups instead of only one.

If the formulae for the acetates were accepted as the most reliable, it was possible to calculate structures for the deacetylated products, hydrolysis of the glucose residue from which would then give the theoretical molecular formulae of the aglycones. This hydrolysis would remove four carbohydrate hydroxyl groups, but would change one oxygen atom in the aglycone into another hydroxyl group, perhaps of a phenolic nature, to leave a net decrease of three. Another oxygen atom in the aglycone corresponded to the methoxyl group. When allowance was made for the latter, the basic structures of the aglycones ranged from $C_{12-13}\ H_{12-14}\ (OH)_4$, including at least one, and possibly two, phenolic hydroxyl groups, to $C_{16}H_{15}(OH)_5$, with at least two or three such groups. These formulae were reminiscent of anthocyanidins $C_{15}H_{10-7}O(OH)_{3-6}$, flavones $C_{15}H_{10-4}O_2(OH)_{0-6}$, and certain other classes of compounds, listed in the Historical Introduction, which occurred naturally as glycosides.

Figure VIII summarized the quantitative aspects of the research, while Figure IX gave the results of a more convenient, but less quantitative separation, in which the adsorption of

FIGURE VIII

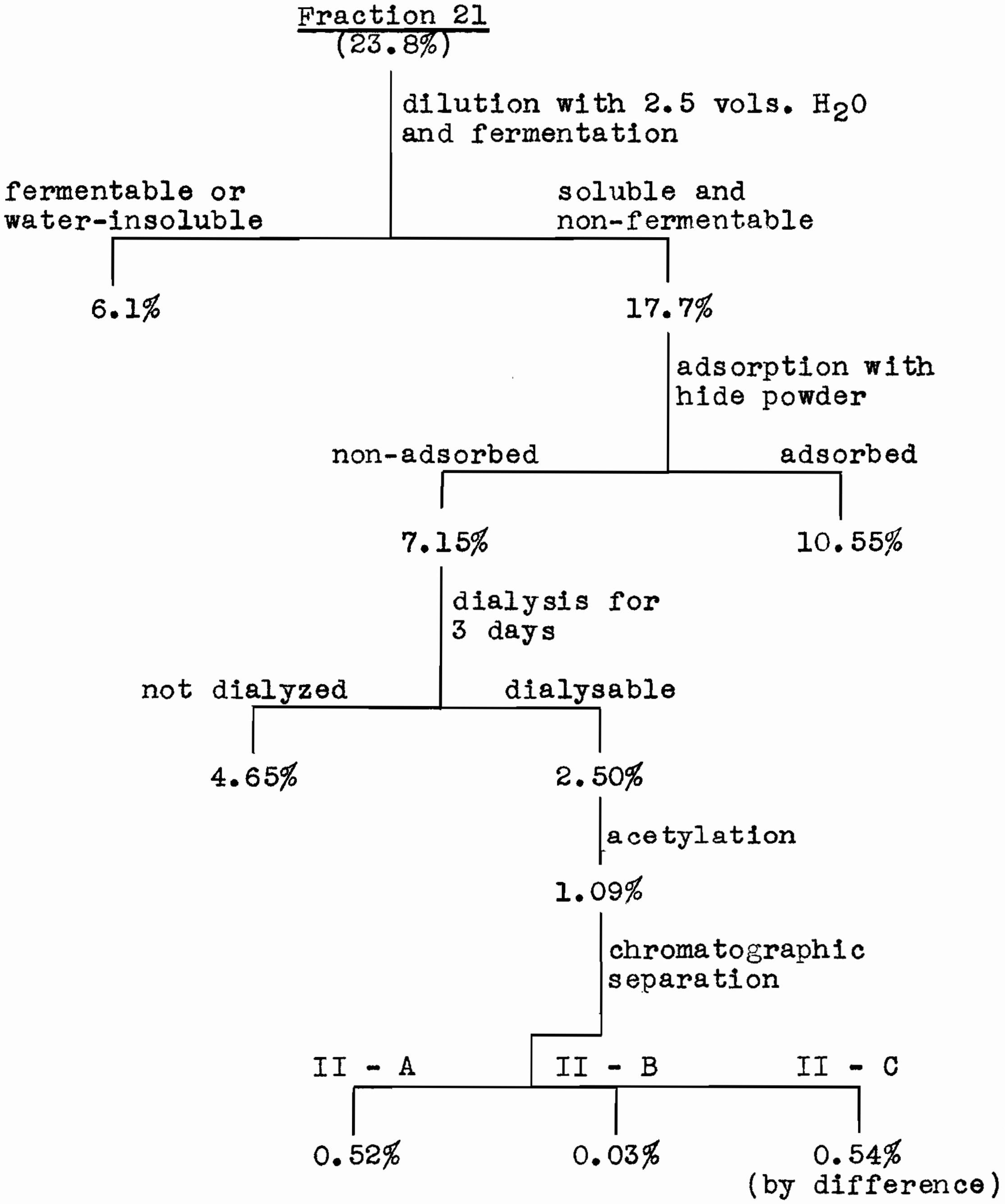
Summary of Separation of Fraction 21[a]



(a) All percentages based on the dry weight of the original bark

FIGURE IX

Summary of Separation of Fraction 21[(a)]



(a) All percentages based on the dry weight of the original bark tannins with hide powder was carried out before, instead of after, a dialysis which was shortened from fifty to three days. The occurrence of fractions I - A and II - A was peculiar because the preliminary examination of the entire original fraction 21 suggested that practically all the β-glucosides had been hydrolyzed by naturally-occurring enzymes. This process had obviously not quite proceeded to completion. It was also very probable that the amount of fractions I - A or II - A in the original bark in non-acetylated form was much greater, and that much of the fermented, dialysable material (8.65%, Fig. VIII) represented the corresponding aglycones. Although attempts to obtain recognizable phenolic substances from the alkaline fusion of this crude material ended in failure, its solubilities and color reactions were reminiscent of those possessed by anthocyanins or flavones. This resemblance extended to the brown-red color assumed by the fermented, dialysable fraction in acidic media, changing reversibly to blue-black in alkali; to the fluorescence shown in alcohol; to the ready solubility in hydroxylic liquids and the insolubility in hydrocarbons; and to a noticeable copper-reducing power. The methoxyl content (4.82%) of this crude fraction corresponded to a substitution of 0.37 to 0.48 in any of the structures tentatively proposed for the aglycones, and acetylation of the remaining hydroxyl groups would yield products with an acetyl content near 35%. The values found were 39.3% and 38.5%.

Although the research failed to establish, or even to separate in pure form, any one definite chemical individual, it established methods of separating the non-tannin components of the dialysable materials, and showed that the aglycones of the mixtures of glycosides isolated were of unexpectedly simple molecular structure. It seemed advisable in future research to employ fresh bark and to inhibit all enzyme action during the extractions with water. The non-tannin components could then be separated by chromatographing the acetates of the glycosides, and the more labile, experimentally less tractable aglycones be isolated by hydrolyzing the individual fractions with emulsin. An alternative method for the separation of glycosidic components from each other, as well as from non-glycosidic constituents, might be that of mixed solvent extraction, as developed by Schudel.

EXPERIMENTAL

Analytical Methods

Copper-Reducing Power

All copper-reducing values were determined by the method of Shaffer and Somogyi (60), which employed a special alkaline copper tartrate solution and a 5-ml. sample containing not more than the equivalent of 2 mg. of glucose. The method was occasionally calibrated, as ml. of 0.005 N thiosulphate against known amounts of pure glucose.

Acid Hydrolysis

The standard conditions for hydrolyzing glycosides consisted of heating the sample in a large volume of 3% aqueous hydrochloric acid at 100° under reflux for 3 hours. After cooling and neutralizing the liquid, any precipitate was removed by centrifuging, and the clear solutions were estimated for copper-reducing power by the Shaffer-Somogyi method. Comparison with the original value gave the percentage, as glucose, of reducing sugars liberated by acid hydrolysis.

Removal of Fermentable Sugars

The aqueous solutions, of concentration 10% or less, were adjusted to pH approximately 4.5 with acetic acid, traces of dihydrogen sodium phosphate and ammonium acetate were added as nutrients, and then small portions of washed baker's yeast. The yeast used also fermented galactose. Fermentation was usually complete after 3 days at approximately 35°. Removal of the yeast by centrifuging permitted a determination of the copper-reducing power, and the difference from the reducing power prior to fermentation was taken as the content of fermentable sugar, expressed as glucose.

Hydrolysis by Emulsin

The cleavage of β-glycosides was carried out in aqueous solution, buffered to pH 4.6 with 0.2 M aqueous sodium acetate - acetic acid solution. Treatment at room temperature with small portions of active emulsin was sufficient for an hydrolysis, which was usually complete within 24 hours. The emulsin was a commercial preparation made by the Eimer and Amend Co., and a 10-mg. sample caused 93% hydrolysis of 15 mg. of pure salicyl-β-D-glucoside in 13 hours.

Pentose

Samples were distilled with 12% hydrochloric acid at a constant rate of 3 ml. per minute for 2 hours, the details given in the standard methods (61) (62) being followed in every particular. The furfural in the distillate was estimated volumetrically by the potassium bromide - bromate method of Powell and Whittaker (61) (62), and the conversion from furfural to pentose was by Krober's tables. It was determined experimentally that the presence of formaldehyde in the distillate had no effect in this estimation.

Galactose

The sample was oxidized with concentrated nitric acid according to van der Haar's method, as modified by Wise and Peterson (63). Any galactose residue present thereby yielded insoluble crystalline mucic acid, which was estimated gravimetrically. Tables by van der Haar were used to convert mucic acid to galactose.

Acetyl

Clark's (64) method of saponification with N ethanolic alkali and subsequent steam distillation of the acidified solution was slightly modified by increasing the amount of distillate collected from 50 ml. to 100 ml., and by ignoring the correction factor.

Nitrogen (Micro Kjeldahl)

White and Long described this method and the reasons for using it (65).

Methoxyl

Methoxyl was determined by Clark's modification (66) of the Vieböck and Schwappach method (67), but the customary scrubbing solution was replaced by a 1:1 mixture of 5% aqueous sodium thiosulphate and 5% aqueous cadmium sulphate, as recommended by Friedrich (68).

Determination of the "Shrink Point"

The increase in the "coagulation" or shrinkage temperature of strips of raw hide, caused by immersion in a solution of the sample as prescribed by the American Leather Chemists Association (10) determined the tanning power and, consequently, the quality of the tannin in the sample.

Adsorption of Tannins by Hide Powder

American Standard Hide Powder was treated according to the standard prescription (9), by digestion in water, and then aqueous chrome alum solution. Tannins were removed by adsorption on the treated hide powder, using a ratio (by weight) of approximately two of treated hide powder to one of sample. This method also provided an estimation of the amount of tannins, by determining the difference in total solids before and after treatment.

Carbon and Hydrogen

These routine estimations were carried out as described in Niederl and Niederl (69), using the "simple band" filling. When this analysis was performed on the deacetylated and methylated fractions, aspiration and wiping of the adsorption tubes were omitted, as recommended by Ogg et al. (70).

Molecular Weight

The Menzies-Wright apparatus enabled the measurement of the difference between the temperature of a boiling alcoholic solution of the sample and the temperature of the vapor at its boiling point (71). The use of an ebullioscopic constant was eliminated by the addition of a standard of known molecular weight (72).

Ash

Niederl and Niederl described the determination used (85). The period of combustion was increased from 10 mins. to 20 mins., and the time of cooling extended to 15 mins.

Ash Alkalinity

The ash was treated with an excess of 0.01 N aqueous sulphuric acid, and the excess acid was titrated with 0.01 N aqueous sodium hydroxide (86). All ash alkalinities were expressed as ml. of N acid per g. of ash.

The Non-Dialysable Fraction 21

Fraction 21, as obtained from Bishop, was a thick, black oil, with a characteristic "woody" aroma. To insure as high a degree of homogeneity as possible for sampling purposes, the material was mixed thoroughly in a Cowles Dissolver, and then stored under toluene. Attempted concentration under vacuum failed because of heavy foaming, which even special anti-foam agents would not break. The solids concentration of the mixture amounted to 57.5% by weight.

In general, hydrocarbon solvents, ether, benzene, and also chloroform, were immiscible with the liquid. "Hydroxylated" solvents, alcohol, water, Cellosolve, and some polar solvents, acetone, pyridine, dioxane, were miscible in varying degree. Solids were precipitated from solution by acids, but not by bases. These observations were summarized in Table III. No water-immiscible solvents were found which would extract solutes from the aqueous mixture.

Tannins

A sample of fraction 21 was diluted with two volumes of water, mixed thoroughly, and the resultant precipitate recovered on the centrifuge. After being washed with a little water, the precipitate was discarded. The combined washings and the dark red-brown, aqueous solution were analyzed for tannin by the standard hide powder test, using 3.2 g. of hide powder. Of the 8.11 g. of solids present in the 50 ml. of solution thus treated, 2.94 g. (36.3%) was adsorbed.

An application of the standard method of the American Leather Chemists Association determined the quality of the tannins. Rawhide strips, measuring 0.5 by 3.5 inches, were tanned by immersion for 48 hours at room temperature in 100-ml. volumes of the above aqueous solution, were washed, dried, and when suspended in water were heated at a uniform rate. Untreated rawhide strips were used as controls. The "shrink point" was 64.5° for the rawhide, and 81.5° for the tanned leather, or an increase of 17°.

Attempted Formation of Basic Salts

An alcoholic solution of fraction 21 could be precipitated with M/2 alcoholic potassium or sodium hydroxide, the light brown precipitate settling out fairly rapidly from the dark brown supernatant liquid. Similarly, alcohol-insoluble amine salts could be formed using diethanolamine, ethanolamine, diamylamine, and dimethylamine, the latter giving the lightest-colored, buff precipitate. Whereas the metal salts were soluble only in water, the amine salts were also soluble in pyridine, although not so in other liquids tried.

Ten grams of fraction 21 (containing 5.7 g. of solids) was dissolved in 100 ml. of ethanol, and 3 ml. of a 33% aqueous solution of dimethylamine was added. The addition of more of the amine produced no more precipitate. After being recovered on the centrifuge, washed with a little ethanol, and dried *in vacuo*, the buff-colored precipitate amounted to 2.6 g. (46%). Heating a 5% solution of the salt in 3% aqueous hydrochloric acid at 100° under reflux for 3 hours resulted in a dark brown insoluble precipitate (68% by weight), while the neutralized filtrate contained copper-reducing material, as glucose, equivalent to 21.6% of the amine salt.

While the dimethylamine salt, either in the solid state or in pyridine solution, showed no fluorescence when irradiated with ultraviolet light, the supernatant alcoholic solution from the amine precipitation, as well as alcoholic solutions of fraction 21, did fluoresce.

Effect of Emulsin

A 12.4-g. sample of fraction 21, containing 7.14 g. of solids, of which 1.50 g. was "glucose", was thoroughly mixed with 100 ml. of water, buffered to pH 4.6 with 0.2 M aqueous sodium acetate - acetic acid, and 0.1 g. of emulsin was added. After 60 hours' stirring at room temperature, the copper-reducing value of the supernatant liquid had not changed. The precipitate and solution were remixed, another 0.1 g. of emulsin was added, and the mixture left at room temperature for an additional 96 hours. Analysis showed 1.55 g. of glucose to be present, representing an increase from 20.2% to 22.0% of the total solids. The activity of the emulsin was tested by adding a small amount of salicin (salicyl-β-D-glucoside) to the mixture and leaving it for 24 hours. A sharp increase in the copper-reducing value of the supernatant liquid occurred.

Other experiments were made using emulsin solutions active enough to hydrolyze 25 ml. of 2% aqueous buffered salicin solution to the extent of 12.6%, 22.0% and 30.8%, in 10, 20 and 30 mins. respectively. Under the same conditions of pH and temperature, however, these solutions failed to increase the copper-reducing value of fraction 21 in periods of up to 72 hours.

An interesting observation resulted during the course of the emulsin experiments. Attempts to cleave crystalline methyl-β-D-glucoside prepared by three different members of this Department by means of the potassium acetate complex (73) with emulsin of proven activity were completely unsuccessful in two cases, and only 9% complete in a third. Samples of β-D-glucosides prepared by the Koenigs-Knorr condensation (74) could be hydrolyzed easily with the same emulsin. Minute traces of some unknown inhibiting substance were presumably present in the former samples.

Analyses for Reducing Sugar

The dilution of 5.20 g. of the original semi-solid fraction 21 concentrate (2.99 g. of solids) caused a dark brown gum to precipitate. After several washings of the insoluble material with small volumes of water, the combined supernatant liquid and washings analyzed for 0.605 g. of glucose (20.2%) by the Shaffer-Somogyi copper-reduction method. Later experiments verified this value.

To determine combined "glucose", 4.8 g. of fraction

21, containing 2.76 g. of solids, including 0.558 g. as glucose, was hydrolyzed with 50 ml. of 3% aqueous hydrochloric acid. During neutralization (pH 6 to 7), the red-brown mixture darkened to blue-black,--a general observation for all acid hydrolyses. Analysis of the filtered solution yielded 1.01 g. as glucose (36.6%), all of which except 0.0228 g. (0.83%) was destroyed by fermentation.

Further analysis (Fig. V) consisted of diluting 22.6 g. (13.0 g. of solids) with 5 volumes of water. This dilution resulted in the precipitation of a dark brown gum. The addition of 3 volumes of water to the brown supernatant liquid caused a further slight precipitation. The gum was recovered with a centrifuge and was washed and dried in vacuo to yield 3.9 g. (30.0%) of a hard, dark brown, amorphous solid. The brown mother liquor contained 9.1 g. of solids (70.0%), of which 2.63 g. (20.2%) was copper-reducing material as glucose. Fermentation of an aliquot of the mother liquor reduced this latter value to an amount corresponding to 8.85%. Acid hydrolysis of another aliquot increased the copper-reducing power to an amount equivalent to 27.6%, as glucose, and fermentation of the neutralized hydrolysate destroyed all but 1.50%. Treatment of a portion of the mother liquor with emulsin for 48 hours increased the "glucose" content to an amount corresponding to 21.2%, as glucose.

It should be noted again that Bishop (5) claimed that dilution as described precipitated 76% of fraction 21, not 25%

and that 24% remained in the mother liquor. A possible explanation of this occurrence is given in the Discussion of Results.

The foregoing work was repeated (Fig. VI) using 20.2 g. (11.6 g. of solids) of fraction 21, and 6 volumes of water for the dilution. The precipitate amounted to 2.97 g. (27.0%), while 8.46 g. (73.0%) remained in solution. Hydrolysis showed 19.5%, 2.26 g., of this amount was "glucose". Duplicate fermentations reduced the amounts of "glucose" in aliquots of the mother liquor to values corresponding to 1.03 g. (8.89%) and 0.945 g. (8.15%). The fermented aqueous solution was extracted in a continuous liquid-liquid extractor with butanol for 36 hours, the operation being carried out at atmospheric pressure, since somewhat elevated temperatures were not expected to affect total solids and copper-reducing values. The alcohol extracted an amount corresponding to 5.35 g. (46.1%), while the aqueous layer retained 2.18 g. (18.8%), of which 0.28 g. (2.4%) was determined to be "glucose". The latter number was increased by acid hydrolysis to a value equivalent to 0.69 g. (5.9%) of "glucose".

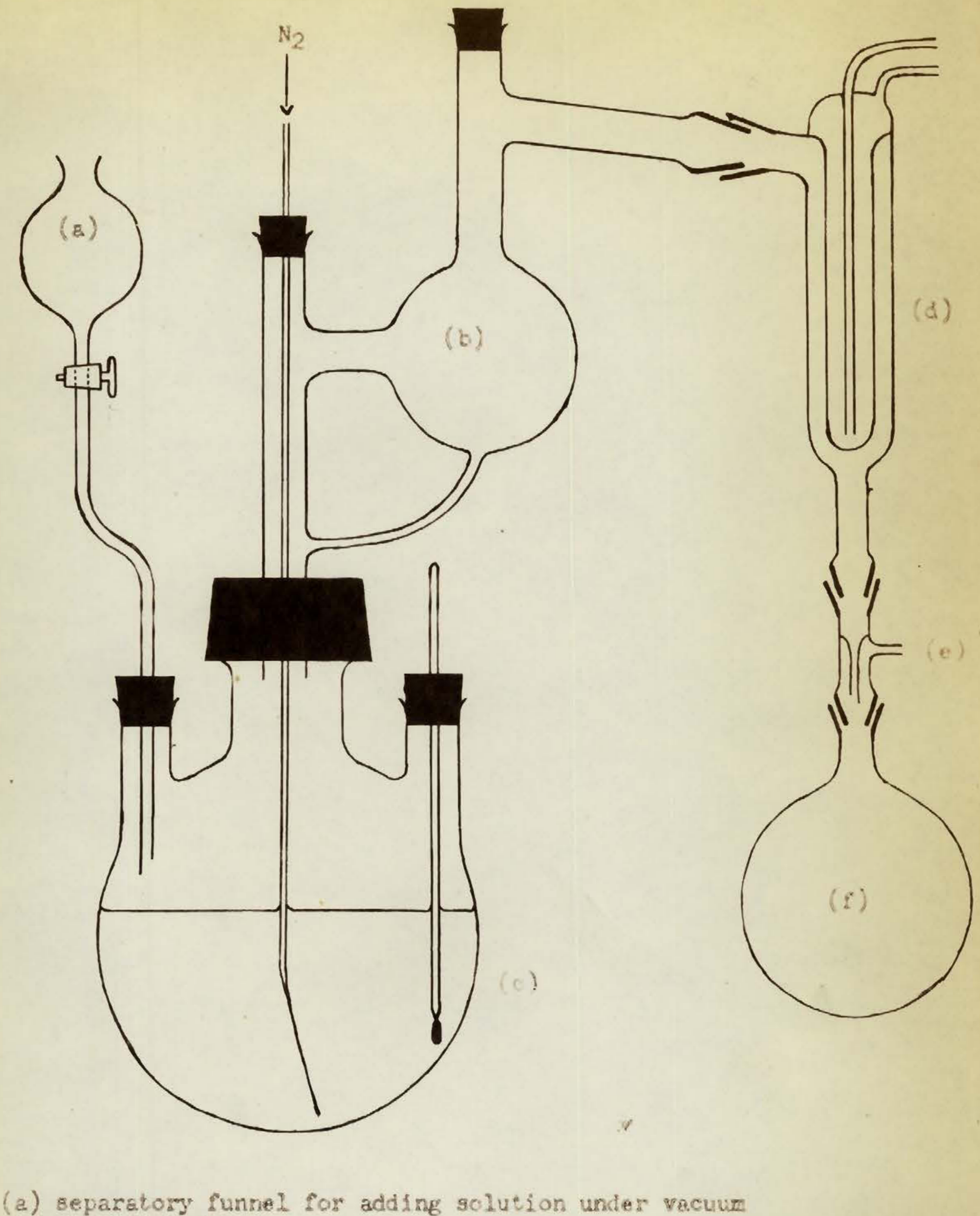
Preliminary Separations by Dialysis

A 22.4-g. sample (12.9 g. of solids) of fraction 21 was dialyzed against distilled water for 72 hours, the dialysate being replaced with fresh water every 12 hours (Fig. VII). At the end of this time, 3.4 g. (by difference), containing 1.69 g. of glucose, had passed through the cellophane membrane. During the dialysis, the dilution of the material inside the sac with water had caused a dark brown gum to precipitate, which was recovered on the centrifuge and was washed and dried (4.0 g.). Acid hydrolysis of the precipitate liberated an additional 0.85 g. of copper-reducing material as glucose. Of the 5.50 g. of solids in the dialyzed solution, 0.86 was "glucose". This latter amount was increased to 1.75 g. of "glucose" by acid hydrolysis.

Large-Scale Precipitation with Water

To minimize errors resulting from operations carried out at different times, a large-scale dilution of 8000 g. (4600 g. of solids) of fraction 21 with 2.5 volumes of water was carried out. The operation, done in batches of 1000 g., was achieved by thoroughly mixing 1 kg. of material with 1700 ml. of water, decanting, and washing the red-brown gummy precipitate with 300 ml. of water. Filtration of the combined washings and mother liquor through Supercel removed any of the precipitate which had been decanted. Extraction of the filter-aid with methanol recovered this material. The water-insoluble gum was also taken up in methanol, and the two alcoholic solutions were combined. The water-insoluble, methanol-soluble gum was not examined further.

To prevent foaming during the vacuum concentration of the aqueous mother liquors, a modified Eastman-Kodak anti-foam device was constructed (75). Its use (Fig. X) with Dow Corning Antifoam Compound permitted the reduction of the volume of the aqueous solution from 22 litres to 9 litres. Nitrogen was



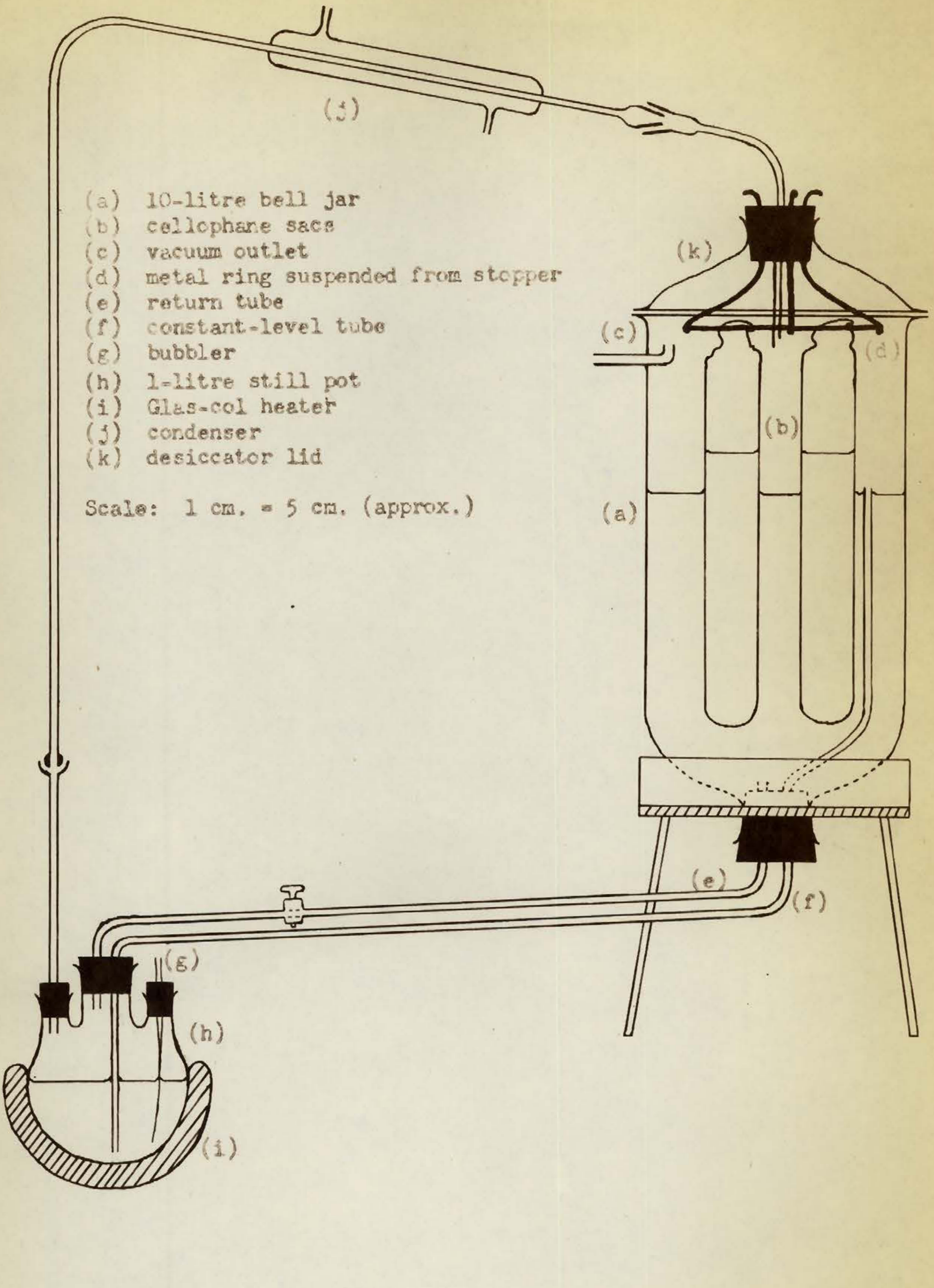
(a) separatory funnel for adding solution under vacuum
(b) anti-foam device with 500-ml. bulb
(c) 2-litre still pot
(d) condenser
(e) vacuum outlet
(f) 1-litre receiver

Scale: 1 cm. = 4 cm. (approx.)

FIG. X Anti-foam Device for Vacuum Concentration bubbled through the boiling mixture, the temperature of which never exceeded 35°, to prevent any oxidation of the substances present. In this manner, a concentrated, dark brown solution containing 3400 g. of solids (74%), with 965 g. (21.0%) as "glucose", was obtained. By difference, 1200 g. (26%) had been precipitated by water.

Large-Scale Dialysis of Fraction 21

A 956-g. portion of the water-soluble material, containing 435 g. of solids, was dialyzed continuously against distilled water under vacuum. The special apparatus for this operation was shown in Fig. XI. Adjustment of the exit flow at (e) to equal the rate of distillation could not be achieved, with the result that the operation was continuous only during the day time. At night, the distillation was stopped, but the dialysis proceeded probably at a slower rate. The apparatus worked very smoothly at a pressure of about 5.5 cm. of mercury, but even at this pressure, the loss of water through the vacuum outlet was appreciable, and more water had to be added from time to time. Sacs for the dialysis were changed every 8 or 9 days. Dilution of the concentrated solution with water during dialysis caused a dark brown gum to precipitate, which was taken up in methanol. After 50 days, the dialysis was discontinued, although an appreciable amount of material was still passing through the membrane. An aliquot of the dialyzed aqueous solution (1.85 g. of solids, of which 0.268 g. was glucose) was further dialyzed. Over a period of one month, 49.5% of the substances in solution appeared in the dialysate, and contained



(a) 10-litre bell jar
(b) cellophane sacs
(c) vacuum outlet
(d) metal ring suspended from stopper
(e) return tube
(f) constant-level tube
(g) bubbler
(h) 1-litre still pot
(i) Glas-col heater
(j) condenser
(k) desiccator lid

Scale: 1 cm. = 5 cm. (approx.)

FIG. XI Vacuum Dialysis Apparatus

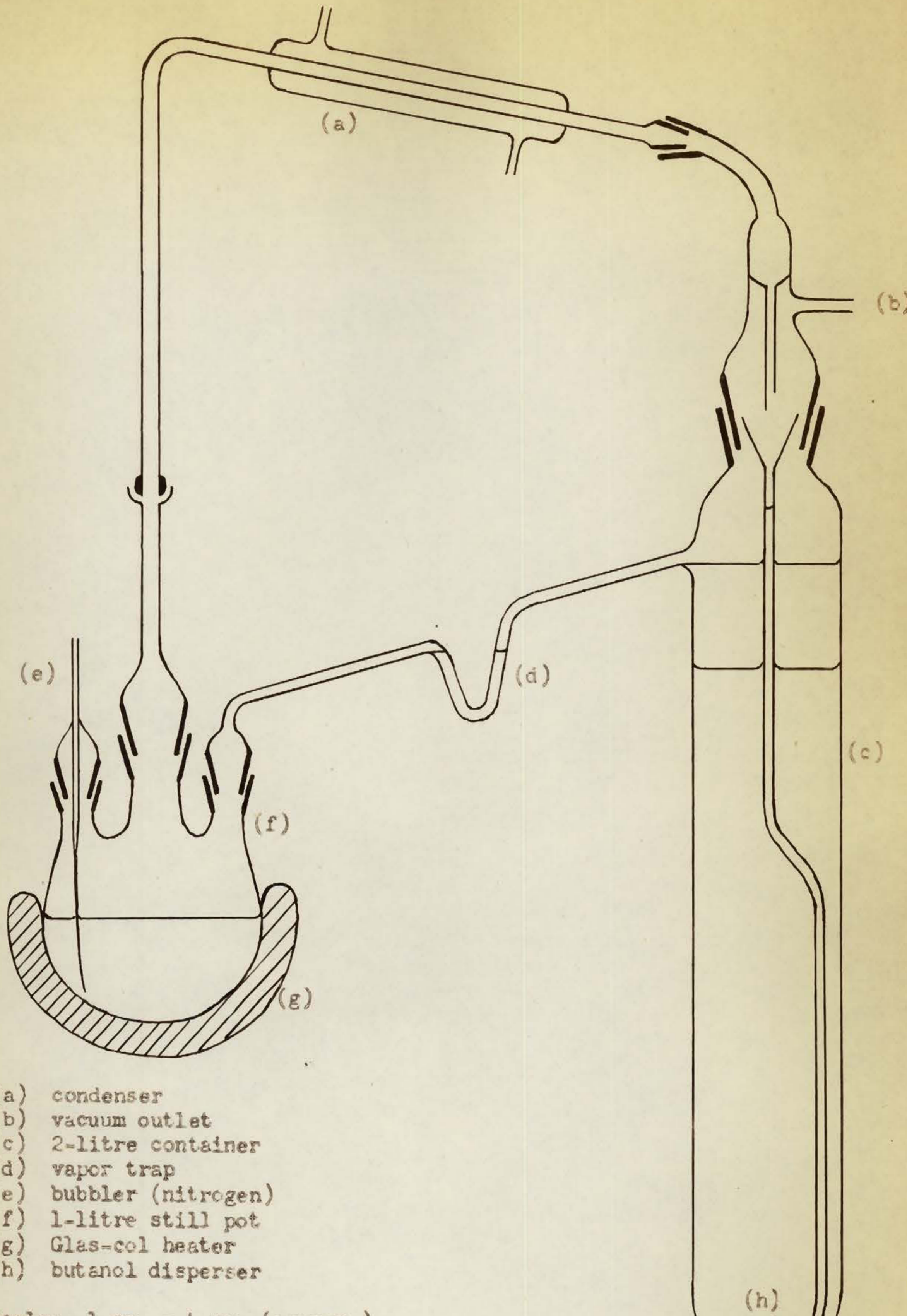
56.7% of the original copper-reducing material in the aliquot. Thus, in all probability, all the water-soluble components of fraction 21 would, in time, have passed through the membrane.

Each batch of amber-colored dialysate was analyzed for copper-reducing power and total solids. After fermentation, these values were re-determined. Sugar values after acid hydrolysis and after fermentation of the neutralized hydrolysates were calculated from work done on aliquots of the fermented dialysates. During the dialysis, 94.4 g. of water-insoluble precipitate was removed from the sacs. Of the original 435 g. of solids, 277.7 g., including 85.0 g. as glucose, had dialyzed. The copper-reducing value was reduced to 27.9 g. by fermentation, and the latter value was increased to 72.6 g. by acid hydrolysis. Fermentation of the hydrolysate destroyed all but 10.29 g. of the "glucose". The data are given in detail in Table IV.

The combined fermented dialysates were taken almost to dryness under vacuum, and left <u>in vacuo</u> over excess phosphoric anhydride for one week. After this time, the viscous brown syrup was stored over anhydrous calcium chloride.

<u>Anal.:</u> <u>Found</u>: galactose, 0.0, 0.0; "furfural", 3.50, 3.33; OCH_3, 4.91, 4.74%.

The aqueous dialyzed solution, containing 63 g. of solids (10.6 g. as glucose) was then extracted under vacuum with butanol in a specially-designed, all-glass extractor (Fig. XII). The conventional liquid-liquid extractor could not



(a)	condenser
(b)	vacuum outlet
(c)	2-litre container
(d)	vapor trap
(e)	bubbler (nitrogen)
(f)	1-litre still pot
(g)	Glas-col heater
(h)	butanol disperser

Scale: 1 cm. = 4 cm. (approx.)

FIG. XII Butanol Extraction Apparatus

be used under vacuum, because of the impossibility of building up a head of butanol sufficient to force this liquid through the aqueous solution, before the head "bumped" or boiled away. After 76 hours of extraction at about 3 cm. pressure, 23.1 g. (5.08 g. as glucose) remained in the aqueous solution, while the alcohol extract contained 51.1 g. The latter solution was concentrated to near dryness under vacuum and the dark brown "gum" was taken up in methanol. Neither solution was investigated further.

Attempted Separation by an Ion-Exchange Resin

As a possible means of fractionating the aqueous mother liquor from the large-scale partial precipitation of fraction 21 with water, chromatography, using Amberlite IRA-400 as the adsorbent, was investigated. This new, strongly basic resin prepared by the Rohm and Haass Co. is capable of adsorbing even weakly acidic compounds, such as phenols. Samples of 1.65 g. and 2.36 g., each in 100 ml. of 25% aqueous methanol, were added to separate columns (25 cm. x 1 cm.) of the resin and the chromatographs were developed with water until the filtrates were colorless. From the solutions, 39.8% of the solids, containing 62.6% of the original "glucose", and 28.9% of the solids, containing 45.1% of the original "glucose", respectively, were removed by the resin. The residues obtained from the filtrates by drying were readily and completely soluble in water, differing in this respect from the original liquor, part of which was precipitated on dilution with water, although not with the 1:3 methanol:water used.

The Fermented, Dialysable Fraction

Preliminary Investigations

The solubility of the fermented, dialysable material corresponded closely to that of fraction 21 itself. Organic liquids, ether, chloroform, petroleum ether, toluene, benzene, ethyl acetate, acetone, dioxane had no effect. The use of pyridine and water, alcohols, and dilute aqueous sodium hydroxide, resulted in complete solutions, those with the first two liquids being yellow, that with alcohol, brown and slightly turbid, and that with alkali blue-black in color. The fractional precipitation of alcoholic solutions, by alcohol-miscible non-solvents such as ether, petroleum ether, benzene, ethyl acetate, gave rise to light-colored, flocculent precipitates, which darkened on drying. The supernatant liquids were light yellow. By way of contrast, dilute alcoholic potassium hydroxide, while precipitating a light brown flocculent material, yielded a dark brown supernatant liquid. After treatment of alcoholic solutions with hydrogen chloride or picric acid, light-colored flocculent materials could be precipitated by ether, but it was not determined whether these solids were identical to, or different from, precipitates thrown down by ether prior to acid treatment. Most of the light-colored precipitates, on being redissolved in alcohol, gave dark brown solutions. The light color thus appeared to be a result of physical state, rather than of purity.

The dark brown precipitate, formed during acid hydrolysis of the fermented dialysables, was soluble in alcohol, and a light brown precipitate could be formed in this solution by the addition of an excess of ether.

To investigate fractionation by means of basic precipitates, 2.24 g. of the fermented dialysables was dissolved in 25 ml. of methanol. The insoluble portion (1.14%), after being removed on the centrifuge and washed with two 7-ml. volumes of methanol, was discarded. The combined washings and dark-brown mother liquor were treated with 35 ml. of M/2 alcoholic potassium hydroxide, centrifuged, and the light-brown, insoluble solid washed with two 50-ml. volumes of methanol, which were added to the alkaline mother liquor (called A). The precipitated alkali-complex, when suspended in 50 ml. of methanol and decomposed with carbon dioxide, underwent a darkening in color as partial solution took place (solution B). After recovery on the centrifuge, the portion that failed to redissolve was washed with methanol, and yielded 0.093 g. of a black amorphous material, which consisted mainly of inorganic substances. The alkaline, alcoholic mother liquor and washings (A) were neutralized with carbon dioxide and centrifuged to remove a light-brown, amorphous solid (0.077g.) which also was chiefly inorganic material. Concentration of the neutralized methanol solutions from (A) and (B) after the removal of the precipitates gave dark-brown, amorphous solids, having pleasant aromatic odors. Further examination of these residues was discontinued.

Attempts were made to degrade the fermented, dialysable fraction in aqueous alkali, with the object of isolating some simple substance, such as catechol, that would give a clue to the chemical type of substances present. A 4.52-g. sample when dissolved in 35 ml. of 15% aqueous sodium hydroxide gave a dark brown, almost black solution, which was heated at 100° under reflux for 2 hours. After being cooled, the solution, on acidification with 19% aqueous hydrochloric acid to pH 1, turned a dark red-brown. The pleasant aroma of the solution when alkaline became disagreeable when acid. After being completely extracted with ether, the acid solution was extracted with ethyl acetate and then with butanol. The removal of these solvents yielded dark brown, amorphous solids, that from the butanol being in the greatest amount. This result was of no particular consequence, as the starting material was soluble in alcohol. The ether solution was back-extracted with 10% aqueous sodium bicarbonate, followed by 5% aqueous sodium hydroxide. Each of these solutions, after being acidified to pH 1 with 19% aqueous hydrochloric acid, was back-extracted with ether. On the removal of the ether, only traces of dark-brown solids were isolated. Repetition of this work under more drastic conditions with 1.83 g. of the fermented dialysable material, 30 g. of potassium hydroxide and 6 ml. of water, in a nickel crucible at 225° for 10 minutes, yielded similar negative results.

Qualitative fractionations by chromatographic adsorption were attempted, using disc chromatograms (84). The adsorbents tested were: Magnesol 5/Celite 1, calcium sulphate, barium carbonate, silicic acid, Florisil, Fuller's earth (with and without Supercel), Bentonite (with and without Supercel), Silene E.F. 2/Celite 1, silica powder, a mixture of equal parts of Magnesol 5/Celite 1 and silic acid, alumina, and calcium carbonate. In all cases, with the exception of alumina, the sample was put on the chromatogram in dilute methanol solution, and developed with 80% aqueous methanol. All the results were either negative or inconclusive; only with silicic acid did separation appear to be occurring. In the latter case, starting from the centre and going out, a colorless ring, a pink-red ring, a light brown ring, a very pale yellow ring, and finally a brown ring, were observed. With alumina, the three initial solvents and eluents used were methanol, water, and 50% aqueous methanol. No separation was observed.

Two columns (25 cm. x 1 cm.) were made up by the wet method (83) using alumina and methanol, and silicic acid 4/Supercel 1 and methanol. Development was with 80% aqueous methanol, 50% aqueous methanol, and finally water. Of the 0.52 g. and 0.46 g. put on these columns, 96% and 99%, respectively, was removed in the first three fractions.

The use of mixed solvents, investigated by Willstätter, and reported by Robinson (16), showed some promise. While ether and diethyl ketone had little or no effect on the fermented, dialysable fraction, ether plus picric acid dissolved small portions, and diethyl ketone plus picric acid much more. In the latter case, the color of the extract was as dark as that of the aqueous solution being extracted. Amyl alcohol or acetophenone, each with or without picric acid, had little solvent power, whereas a mixture of amyl alcohol, acetophenone and picric acid was an extremely good extracting medium, leaving the aqueous solution colored yellow. These qualitative investigations were not carried any further. This method of mixed solvent extraction has been used for the separation of anthocyanins in plants, ether - picric acid extracting the sugar-free anthocyanidins, diethyl ketone - picric acid extracting the monoglucosides, and acetophenone - amyl alcohol - picric acid, the diglucosides (76)(77).

Methylation of the fermented, dialysable fraction was carried out by dissolving a 1.18-g. sample in 6 ml. of anhydrous methanol. A small amount of insoluble material (1.41%), after removal on the centrifuge, was discarded. Following treatment of the dark red-brown, alcoholic solution with approximately 1 g. of diazomethane at 0° - 5°, and standing for 12 hours at 5°, any excess diazomethane was removed by heating the solution to 60° for 30 mins. Distillation _in vacuo_ removed the solvent, and the residual syrup was extracted with boiling petroleum ether. Drying the residue under vacuum, about 1 mm., for 48 hours caused the syrup to "fluff up", after which it was ground in a mortar to a sparkling orange-brown powder. The methylated material (1.10 g.), was soluble in methanol, acetic acid, pyridine, but only partially soluble in water or dioxane.

Anal.: Found: OCH_3, 15.5, 16.3%.

Remethylation was effected in dioxane suspension with approximately 1 g. of diazomethane. The product was recovered by concentration and extraction with chloroform. Solution in this solvent was not complete, but nearly so. The methylated product was precipitated with excess ether, washed with petroleum ether, and dried *in vacuo*. The yield was not quantitative because of a mechanical loss.

Anal.: Found: OCH_3, 19.6, 19.7%.

Acetylation of the fermented, dialysable fraction consisted of dissolving a 5.12-g. portion in 25 ml. of anhydrous pyridine, which was the minimum necessary for solution, and adding 40 ml. of acetic anhydride to the dark brown solution. Heat was liberated almost immediately and the temperature of the reaction mixture rose to 45° to 50°. The mixture was allowed to cool naturally to room temperature, and left standing for 2 hours. The addition of 350 ml. of water precipitated a light-brown, oily material. Rapid stirring for 24 hours changed this oily precipitate to a hard, light-brown solid, which was recovered on a filter, triturated with water, and dried *in vacuo*. Yield, 6.28 g.

Anal.: Found: acetyl, 35.4, 36.2%.

A reacetylation of 6.09 g. with 25 ml. of acetic anhydride, 2 ml. of anhydrous pyridine, at 100° under reflux for 1 hour, led to 5.44 g. of a product which was soluble in a wide range of organic solvents, but was insoluble in water, ether and petroleum ether.

Anal.: Found: acetyl, 39.8, 38.8; OCH_3, 3.21, 3.35%.

A 10.51-g. sample of the fermented, dialysable fraction was acetylated in 80 ml. of acetic anhydride and 35 ml. of anhydrous pyridine at 100° under reflux for 2 hours. Precipitation with 700 ml. of water, and recovery of the product in the usual manner, led to a yield of 12.93 g. of a light buff-colored solid.

Anal.: Found: acetyl, 38.7, 38.2; OCH_3, 3.36, 3.38%.

Nierenstein suggested a method for the conversion of phenolic acetates to methoxyl derivatives (78). In accord with this method, a 1.94-g. sample of the acetate (acetyl 39.8, 38.8%) was dissolved in 20 ml. of anhydrous dioxane, and 1.8 ml. of anhydrous piperidine was added. The molar ratio of acetyl groups to piperidine was 1:1. The dioxane-piperidine solution was treated with approximately 1 g. of diazomethane. After standing at 5° for 12 hours, the solution was acidified to pH 5-6 with 0.5 N aqueous hydrochloric acid, and completely extracted with chloroform. The chloroform solution was back-extracted with two 10-ml. volumes of water, dried over sodium sulphate, and concentrated *in vacuo*. The addition of excess ether to the chloroform extract precipitated a light-brown solid, which was recovered by filtration and extracted with petroleum ether. The yield was not determined.

Anal.: Found: OCH_3, 5.76, 5.86; acetyl, 29.1, 29.8%.

Similar treatment of a 2.05-g. sample of the acetate (acetyl, 38.7, 38.2%) led to 1.90 g. of product.

Anal.: Found: OCH_3, 4.99, 5.00; acetyl, 27.8, 27.1%.

Rao et al described the conversion of flavone acetates to the corresponding methoxyl compounds (79, 80, 81). A solution of 2.79 g. of the acetate (acetyl, 39.8, 38.8%) in 120 ml. of acetone was treated with 30 ml. of dimethyl sulphate and 30 ml. of 25% aqueous sodium hydroxide, followed by the alternate addition of six 5-ml. volumes of dimethyl sulphate and six 10-ml. volumes of 25% aqueous sodium hydroxide. The reaction flask was cooled in an ice-bath, during the addition which lasted 12 minutes. Since the reaction mixture remained heterogeneous, the ice-bath was replaced with a warm water bath at 45°. Ten minutes later refluxing began, and 3 to 4 mins. afterwards, the mixture became homogeneous. The bath was removed and stirring at room temperature continued for 7 hours. Evaporation of the acetone under vacuum caused the precipitation of a brown gum, which was taken up in chloroform. The aqueous solution was also extracted with chloroform. The product was isolated from the combined chloroform solutions as described in the previous Section. The yield (1.04 g.) of the light brown powder could not be determined quantitatively because of accidental loss.

Anal.: Found: OCH_3, 27.7, 28.4%.

A slightly different treatment of 2.01 g. of the acetate (acetyl 38.7, 38.2%) consisted of alternate addition of two 15-ml. volumes each of dimethyl sulphate and 25% aqueous sodium hydroxide, followed by five 5-ml. volumes each of dimethyl sulphate and the alkali, while gentle refluxing was maintained with a warm water bath. At the end of the addition, 30 minutes later, 15 ml. of 25% aqueous alkali was added. Working up the reaction mixture led to the recovery of 0.91 g. of a light-brown, amorphous solid.

Anal.: Found: OCH_3, 33.8, 34.1%.

Analysis of the remaining aqueous solution showed a negligible copper-reducing power.

A 5.40-g. sample of the fermented, dialysable fraction was hydrolyzed in 50 ml. of 3% aqueous hydrochloric acid at 100° under reflux for 2.5 hours. The centrifuge removed 1.87 g. of dark-brown precipitate and neutralization with aqueous alkali precipitated an additional 0.10 g. of a brown-red solid, which was also removed. The aqueous brown-red filtrate was fermented with yeast for 4 days, the yeast removed on a filter, and the solution taken to dryness *in vacuo*. Anhydrous pyridine (10 ml.) served to extract the aglucones from the syrup, and the extract was heated with 20 ml. of acetic anhydride at 100° under reflux for 3 hours. The dark-brown, water-insoluble product amounted to 0.65 g.

Anal.: Found: acetyl, 38.3, 38.1%.

Removal of Tannins using Hide Powder; Acetylation of Non-Adsorbed Material.

Two aqueous solutions of the fermented, dialysable fraction, containing 2.75 g. in 60 ml., and 1.84 g. in 40 ml., were separately mixed with 3.1-g. portions of treated hide powder, which was approximately 2 years old, for 10 mins., filtered, and the total solids concentration of the filtrates determined. The amounts adsorbed by the hide powder were 1.29 g. (47.0%) and 0.89 g. (48.5%), respectively. The different amounts of material adsorbed by the same amount of hide powder were deemed sufficient evidence that maximum adsorption had occurred. Much color was also removed from the aqueous solutions. The filtrates were combined and concentrated *in vacuo* to dryness.

Anal.: Found: methoxyl, 6.39, 6.40%.

A 0.51-g. sample of the non-adsorbed material was acetylated with 2 ml. of anhydrous pyridine and 5 ml. of acetic anhydride at 100° for 1.5 hours. Yield, 0.63 g.

Anal.: Found: acetyl, 40.6, 40.8%.

Acetylation of 0.88 g. in proportionately greater amounts of reagents at room temperature for 2.5 hours resulted in 1.00 g.

of a buff-colored, amorphous solid.

Anal.: Found: acetyl, 39.6, 40.1%.

These acetates were soluble in most organic solvents, but insoluble in water, ether, petroleum ether and carbon tetrachloride.

Preliminary Investigation of the Acetates

Distillation of the crude acetate mixtures of either the fermented dialysables or of the material not adsorbed by hide powder was unsuccessful. At pressures as low as 8 microns and bath temperatures as high as 225°, no material distilled. The acetates liquified between 100° and 150°, and charred considerably over a 2-hour heating period.

Chromatographic separations on alumina (Brockmann standardization, grade II or III (82)) showed great promise. Several small-scale runs, using approximately 30 g. of alumina in columns (25 cm. x 1 cm.) packed by the wet method (83), were carried out. Acetate samples of about 0.5 g. were put on the columns in 10% anhydrous benzene solutions. When elution with benzene was complete, gradual increments of methanol in benzene removed further amounts of material. Low concentrations of acetic acid in methanol (2 to 5%) usually completed the recovery. In all cases it was found that approximately 50% passed through the column in benzene; 5% to 10% methanol in benzene recovered an additional 30%, while the acetic acid -

methanol removed the remainder. Qualitatively, it appeared that the separation of the acetates of the material not adsorbed by hide powder was more successful than of the acetates of the original fermented, dialysable fraction, which had not been treated with hide powder.

First Large-Scale Hide Powder Adsorption; Acetylation of Non-Adsorbed Material.

A 129-g. portion of the fermented, dialysable fraction was mixed with 250 g. of treated hide powder as prescribed. Concentration of the aqueous filtrate in vacuo to dryness, and a further period of dehydration under a vacuum of approximately 1 mm., at 35°-40° for 48 hours, led to the recovery of 51.5 g. (39.8%) of a dark brown syrup. Acetylation with 250 ml. of anhydrous pyridine and 700 ml. of acetic anhydride at room temperature for 3 hours, and recovery of the acetate in the usual manner, yielded 52.3 g. of a cream-colored, amorphous solid.

Anal.: Found: acetyl, 39.6, 39.2%.

The aqueous acid filtrate from the acetylation was neutralized with caustic soda and concentrated in vacuo at temperatures always below 50°. As the concentration proceeded, sodium acetate crystallized, and was removed from time to time by filtration. The residue from the distillation was extracted with 200 ml. of 1:1 pyridine-ethanol, and the extract evaporated to leave 12.5 g. of a light orange-brown, amorphous solid, which was soluble in water, methanol, acetic acid, and acetic anhydride.

Anal.: Found: ash, 49.3, 49.6%.

Reacetylation in 100 ml. of acetic anhydride and 5 ml. of anhydrous pyridine for 2 hours at 40° failed to produce any water-insoluble material, and the investigation was discontinued.

Attempted Chromatographic Separation of the Acetate

A chromatographic adsorption column, of length 92 cm., and inner diameter 3.80 cm., was made up using approximately 900 g. of alumina and anhydrous benzene, by the wet method (83). The alumina, purchased from the Harshaw Chemical Co. of Cleveland, under the label AL 0109 P, was determined to be of Grade I, according to the Brockmann standardization (82). A 23.0-g. sample of the acetate (acetyl 39.6, 39.2%) was dissolved in 300 ml. of anhydrous benzene, and put on the column. Elution with benzene, methanol in benzene (2% to 75%), methanol, pyridine in benzene (2% to 10%), recovered only negligible amounts of material. Recovery of the adsorbent as sections approximately 8 cm. long, and extraction of each section first with pyridine, then with 50% alcoholic acetic acid, and finally with acetic acid, removed less than 5% of the adsorbed material. It appeared that the acetate was irretrievably adsorbed on the alumina, and the run had to be discontinued.

Chromatographic Separation of the Acetate, Run I.

The column, of length 92 cm. and inner diameter 3.80 cm., was made up by the wet method (83), using 970 g. of alumina

(Brockmann Grade III) and anhydrous benzene. A 29.0-g. sample of the same acetate was dissolved in 250 ml. of anhydrous benzene and put on the column. The flow rate through this column was approximately 350 ml. per hour. Development of the column and other data are given in Table VII. Combination of the various fractions was as follows: fractions 4, 5, 6, 7 combined to give I - A (an amber syrup), fractions 8, 9, 10, 11, 12 combined to give I - B (an amber syrup), fractions 21 to 27, inclusive, combined to give I - C (a light brown solid), and fractions 29 to 36, inclusive, combined to give I - D, a light brown, crystalline solid.

Fraction I - C, 2.51 g., after the removal of the solvent, was only slightly soluble in methanol, but nearly completely soluble in water. Solution in water, filtration of the insoluble material, and removal of the solvent <u>in vacuo</u>, yielded 2.06 g. of a buff-colored amorphous solid.

<u>Anal.</u>: <u>Found</u>: ash, 64.9, 64.3%; ash alkalinity, 5.90, 6.18; $[\alpha]_D^{25°}$, 0° (c = 1.66, water).

No further investigation of this fraction was carried out.

Fractions I - A, I - B, and I - D, will be discussed later in conjunction with fractions from Run II.

TABLE VII

CHROMATOGRAPHIC SEPARATION OF ACETATES, RUN I

Solvent	Fraction (250 ml.)	Color of Fraction	Weight of Residue (g.)	
benzene	1	colorless	...	
	2 and 3	colorless	...	
5% methanol in benzene	4	very pale yellow	3.29	13.81 g. (47.6%)
	5	very pale yellow	8.70	
	6	colorless	1.36	
	7	colorless	0.46	
	8	very pale yellow	1.48	9.60 g. (33.1%)
	9	deep yellow	6.48	
	10	yellow	1.36	
	11	pale yellow	0.17	
	12	very pale yellow	0.11	
25% methanol in benzene	13	colorless	...	
	14 to 17 inc.	colorless	...	
methanol	18 to 20 inc.	colorless	...	
	21	very pale yellow	0.41	2.51 g. (8.6%)
	22	very pale yellow	0.84	
	23	very pale yellow	0.51	
	24	colorless	0.28	
	25	colorless	0.21	
5% acetic acid in methanol	26	colorless	0.15	
	27	colorless	0.11	
	28	colorless	...	
	29	pale yellow	0.23	10.05 g. (34.6%)
	30	dark yellow-brown	5.00	
	31	yellow-brown	1.92	
	32	light yellow	1.00	
	33	light yellow	0.72	
	34	pale yellow	0.47	
	35	very pale yellow	0.44	
	36	colorless	0.27	

Fermentation; Adsorption by Hide Powder; Dialysis; Acetylation of a New Batch of Material.

A 1075-g. portion of the water-soluble part of the original fraction 21, containing 490 g. of total solids, of which 139 g. was "glucose", was precipitated with 5 litres (roughly 2.5 volumes) of water, and the aqueous mixture was fermented with 40 g. of baker's yeast, in the presence of 2 g. of dihydrogen sodium phosphate, 2 g. of ammonium acetate, and 10 ml. of glacial acetic acid. Filtration with the aid of Supercel removed the water-insoluble material and the yeast. The dark red-brown filtrate contained 364 g. of solids (74.3%), and was copper-reducing to the extent of 63.4 g. of glucose.

Tannins were removed from a portion of the solution, containing 354 g. of solids, with 500 g. of treated hide powder, and the aqueous residue of non-adsorbed material was concentrated to about 350 ml. Total non-adsorbed solids amounted to 143 g. (40.4%).

The concentrated aqueous solution was continuously stirred while it was being subjected to dialysis against distilled water at atmospheric pressure for 3 days, and the dialysate was also stirred intermittently. The dialysate was replaced with fresh water twice daily. Concentration of the combined amber-colored dialysates *in vacuo* led to the recovery of 50.0 g. (35.0%) of a dark brown syrup.

Acetylation with 350 ml. of anhydrous pyridine, and

800 ml. of acetic anhydride at room temperature for 2 hours, and recovery of the product in the usual manner, gave 36.4 g. of a buff-colored amorphous solid.

Anal.: Found: acetyl, 40.7, 41.2%.

Chromatographic Separation of the Acetate, Run II.

The column was prepared, as already described, using 1100 g. of alumina (Brockmann Grade III). A 36.2-g. portion of the acetate (acetyl 40.7, 41.2%) in 300 ml. of anhydrous benzene was put on the column at a flow rate of approximately 370 ml. per hour. The data for this run were given in Table VIII. Fractions 13 to 17, inclusive, were combined to give II - A, an amber syrup; fractions 30 to 34, inclusive, gave II - B, a light-brown solid; fractions 35 to 38, inclusive, gave II - C, a light-brown, crystalline solid; fractions 39, 40, 41 gave II - D, a brown, gelatinous solid.

Fraction II - B, while only slightly soluble in methanol, was completely soluble in water (cf. fraction I - C), and was not investigated further. Fraction II - D was insoluble in methanol, appeared to be inorganic in nature, and was discarded without further analysis.

Investigation of Fractions I - D and II - C

The inadvertent mixing of these fractions during the initial investigations necessitated their investigation as a unit. The fractions, as originally obtained after the removal

TABLE VIII

CHROMATOGRAPHIC SEPARATION OF ACETATES, RUN II

Solvent	Fraction (250 ml.)	Color of Fraction	Weight of Residue (g.)	
benzene	1 to 5 inc.	colorless	...	
5% methanol in benzene	6 to 10 inc.	colorless	...	
10% methanol in benzene	11 and 12	colorless	...	
	13	very pale yellow	5.54	17.09 g. (47.2%)
	14	very pale yellow	6.88	
	15	very pale yellow	2.63	
20% methanol in benzene	16	colorless	1.28	
	17	colorless	0.76	
	18 to 20 inc.	colorless	...	
50% methanol in benzene	21 to 26 inc.	colorless	...	
methanol	27 to 29 inc.	colorless	...	
	30	colorless	0.13	1.09 g. (3.0%)
5% acetic acid in methanol	31	colorless	0.24	
	32	colorless	0.25	
	33	colorless	0.24	
	34	colorless	0.23	
	35	yellow	4.72	27.95 g. (77.2%)
15% acetic acid in methanol	36	yellow	12.76	
	37	pale yellow	7.18	
	38	very pale yellow	3.29	
	39	very pale yellow	6.76	21.90 g.
	40	very pale yellow	10.63	
	41	very pale yellow	4.51	

of the solvent, were crystalline. The colors of the various sub-fractions varied slightly, but in general, all were light brown. Various solvents were tested as means of purification. Methanol and ethanol readily gave very dark brown solutions, with a small amount of insoluble material, but crystallization could be induced only in very concentrated solutions, and filtration of the resultant "sludge" was very difficult. The cooling of more dilute methanol solutions to as low as -50° did not promote crystallization. The higher alcohols, i.e. amyl, hexyl, their branched-chain analogues, cyclohexyl, and benzyl, were solvents only at higher temperatures. Once in solution, the substance was not precipitated on cooling. Nitrobenzene and 2-nitropropane were non-solvents, as were all the common hydrocarbon liquids. Attempts to precipitate the material from alcohol solution with most non-solvents tried led to amorphous, brown, flocculent solids, with nearly colorless supernatant liquids. On the evaporation of 10% alcoholic benzene extracts of the material, however, very fine, nearly colorless needles were deposited.

Deacetylation of 0.50 g. of the impure crystals in 3 ml. of anhydrous methanol was attempted by the addition of 15 ml. of methanolic sodium methoxide. This amount of deacetylating agent, approximately 0.004 mole, was necessary to retain the alkaline reaction toward phenolphthalein observed when a few drops of the reaction solution was diluted with water. After 24 hours, the solution was still alkaline to phenolphthalein.

Crystallization did not occur until nearly all the solvent had been removed, and even then the formation of crystals was indefinite. The addition of water to this concentrated alcoholic solution precipitated nothing. Ether, benzene, dioxane, acetone all gave rise to flocculent, brown, amorphous solids. Complete removal of the alcohol left a residue which had the odor of methyl acetate, and an examination under the microscope gave no definite evidence of the presence of crystals.

Purification by re-chromatographing the acetylated mixture met with no success. Of the 0.48 g. in 10 ml. of methanol put on a small column, containing approximately 30 g. of alumina, and developed with 2% acetic acid in benzene, the residues from the first 9 fractions were 0.02 g., 0.09 g., 0.06 g., 0.06 g., 0.07 g., 0.07 g., 0.08 g., and 0.03 g., respectively, or over 100% recovery, with no separation.

The 30 g. of fractions I - D and II - C remaining from these investigations was taken up in 50 ml. of anhydrous methanol, and 7.16 g. of a brown amorphous solid was removed by filtration. The addition of 450 ml. of benzene to the alcoholic solution gave a dark-brown, granular precipitate immediately. After 5 minutes of stirring, the mixture was allowed to settle, and was filtered (filtrate 1). The precipitate was redissolved in 50 ml. of anhydrous methanol and reprecipitated as before. Filtration gave filtrate 2, and a dark brown residue (A), which was dried *in vacuo* (16.3 g.). After 48 hours at half an atmosphere pressure over anhydrous calcium chloride, paraffin and solid sodium hydroxide, filtrates 1 and 2 deposited solids B (0.83 g.) and C (0.72 g.), respectively, which appeared crystalline. Since further evaporation of the separate filtrates caused no further precipitation, they were combined, and the concentration continued. When nearly all the solvent had been removed, 1.2 g. of a granular solid (D) was obtained, and complete removal of the solvent led to 1.5 g. of a brown, amorphous solid (E).

The identity or non-identity of the solids B, C, D, E was investigated by three different means. If saturated solutions of two solids in the same solvent are mixed, the appearance of "schlieren", or mixing lines, indicates non-identity (87). Using micro test tubes of length 40 mm., inner diameter 2 mm., and oblique illumination, "schlieren" were observed on mixing saturated methanolic solutions of every possible combination, except C with E. The next test showed that B, C, D, E were all optically inactive, as determined on 2% methanolic solutions of each. In the third method, B sintered at 151° and melted with decomposition at 155.5° - 158.0°, C charred slightly at 300°, with no further change up to 360°, D sintered at 99° and decomposed at 142° - 147°, E sintered at 90° and melted at 120.5° - 124.5° with decomposition, all temperatures being corrected. Consideration showed that the results of the three tests failed to establish the identity of any one fraction with another. It was eventually found that all the subfractions had large ash contents (Table IX), which probably originated in substances stripped from the alumina by the acetic acid - methanol solutions used to elute acetates I - D and II - C.

TABLE IX

ASH CONTENTS AND ASH ALKALINITIES OF VARIOUS SUBFRACTIONS

Subfraction	Ash (%)		Ash Alkalinity[(a)]	
A	36.4,	35.9	12.0 ,	12.1
B	38.6,	37.8	17.8 ,	19.1
C	45.6,	45.0	17.9 ,	17.6
D	51.6,	51.0	2.98,	3.04
E	18.5,	19.1	9.75,	10.3

(a) Expressed as ml. of N acid per g. of ash. Since 106 g. of Na_2CO_3 require 2 litres of N acid, the value for Na_2CO_3 is $\frac{2000}{106}$, or 18.9.

Investigation of Fraction I - A

I - A was readily dissolved in 40 ml. of methanol. A trace of insoluble material was removed by filtration, and 500 ml. of water added to precipitate the acetate. While the bulk of the material settled out rapidly, the supernatant liquid remained opalescent. Acetic acid, 10 ml., clarified the liquid, and after 24 hours the mixture was filtered, and the solid ground with water in a Waring Blendor. Filtration and drying *in vacuo* recovered the acetate as a buff-colored, amorphous powder. The aqueous acid filtrate was shown to contain only negligible traces of residue, by concentration *in vacuo* to dryness.

Anal.: *Found*: Ash, 0.0, 0.0; acetyl, 39.1, 39.2; nitrogen 0.0, 0.0%; $[\alpha]_D^{25°}$, - 20.0° (c = 1.604, methanol).

A 4.7-g. sample of I - A was re-acetylated in 25 ml. of acetic anhydride and 2 ml. of anhydrous pyridine at room temperature for 1 hour, and then at 40° for 1 hour. The recovery of the product, in the usual manner, led to a 98% yield of a nearly-white, amorphous solid.

Anal.: *Calcd.* for $C_{16}H_{16}O_7(C_2H_3O)_5(OCH_3)$: C, 57.23; H, 6.05; acetyl, 37.9; OCH_3 5.46%; Mol. Wt., 567.

Calcd. for $C_{18}H_{17}O_8(C_2H_3O)_6(OCH_3)$: C, 57.20; H, 5.89; acetyl, 39.7; OCH_3, 4.76%; Mol. Wt., 651.

Calcd. for $C_{18}H_{19}O_8(C_2H_3O)_6(OCH_3)$: C, 57.06; H, 6.18; acetyl, 39.6; OCH_3, 4.75%; Mol. Wt., 653.

Found: C, 57.22, 57.14; H, 6.00, 6.09; acetyl, 40.5, 40.1; OCH_3, 3.72, 3.61%; $[\alpha]_D^{25°}$ -19.6° (c = 1.785, methanol); Mol. Wt., 602, 645 (benzil as standard), 593, 661 (sucrose octaacetate as standard).

Deacetylation was accomplished using 0.425 molar methanolic barium methoxide, prepared according to directions given by Mitchell (88). A 7.21-g. sample of a mixture of I - A and reacetylated I - A was dissolved in 75 ml. of anhydrous methanol to give a yellow solution. Five ml. of the barium methoxide solution was sufficient for the deacetylation, as determined by removing a few drops of the reaction mixture at intervals over a 24-hour period, diluting with water, and testing with phenolphthalein. Immediately upon the addition of the barium methoxide, the color of the solution changed from yellow to dark brown, and a dark brown solid precipitated. These conditions persisted throughout the deacetylation. After 24 hours, 100 ml. of water was added to give a clear dark brown solution. Barium was removed by the addition of 0.9 N aqueous sulphuric acid and finally 0.05 N aqueous sulphuric acid (dropwise), until a few drops of the supernatant liquid, on being added to either dilute aqueous sulphuric acid or dilute aqueous barium hydroxide, gave no observable precipitation. The mixture was filtered through a pad of Supercel (pH of filtrate approx. 3), the solution concentrated to dryness *in vacuo*, and the residual brown syrup dissolved in 15 ml. of methanol. Precipitation with 200 ml. of ether recovered the product as a light brown solid, which was extracted with pentane, and dried *in vacuo* (yield, 2.00 g.). The ether-alcohol filtrate contained no residue.

Anal.: *Found*: ash, 4.11, 4.07%.

A 0.0456-g. sample (corrected for ash) of deacetylated I - A was subjected to hydrolysis in 25.0 ml. of 3% aqueous sulphuric acid. Cooling, filtration to remove the insoluble material and any barium sulphate, neutralization and filtration, led to an amber filtrate, which was fermented with baker's yeast. The copper-reducing powers before acid hydrolysis, after acid hydrolysis, and after fermentation were 0.0979, 0.517, and 0.0769 g. as glucose, per g. of ash-free material, respectively.

Anal.: Calcd. for the expected deacetylated monoglucoside: Mol. Wt., 398.

Found: 1 mole "glucose" per Mol. Wt. of 430, 410.

Deacetylated I - A, 1.79 g., was dissolved in 30 ml. of anhydrous methanol, and some insoluble material (0.054 g.) removed by filtration. The bulk of the filtrate was lost through an accident, but about 1/3 was saved for subsequent analysis. Precipitation with excess ether, filtration, and prolonged extraction of the residue with pentane yielded 0.49 g. of a light brown, amorphous solid.

Anal.: Calcd. for $C_{17}H_{23}O_8(OCH_3)$: C, 55.94; H, 6.80; OCH_3, 8.01%; Mol. Wt., 387.

Found: Ash, 4.74, 4.54; C, 53.56, 53.59; H, 6.58, 6.50; OCH_3, 9.87, 9.88%.

Corrected for ash: C, 56.15, 56.20; H, 6.90, 6.83; OCH_3, 10.4, 10.4%.

Hydrolysis of 0.0595 g. of deacetylated I - A, on an ash-free basis, for 24 hours with 30 mg. of emulsin increased the copper reducing power from 0.128 g. to 0.228 g. as glucose, per g. of ash-free material. The addition of more emulsin for a further period of 48 hours changed the latter value to only 0.230 g. A small amount of salicin was then added. In 4 hours, the copper-reducing power of the solution had risen markedly, showing the emulsin to still be active.

A 0.25-g. portion of deacetylated I - A was treated in 10 ml. of anhydrous methanol with two 0.5-g. amounts of diazomethane, added 24 hours apart. The diazomethane was swept from the producer flash with a stream of nitrogen through a reflux condenser and bubbled into the methanol solution. The carrying over of some ether with the diazomethane caused partial precipitation in the reaction flask. After a further 24 hours at 5°, the reaction mixture was concentrated _in vacuo_, and the residue extracted with pentane to give an orange-brown amorphous solid (0.25g.)

Anal.: _Found_: ash, 4.91; OCH_3, 16.6, 16.2%.

Corrected for ash: OCH_3, 17.4, 17.0%.

A third methylation with 0.5 g. of diazomethane, and recovery of the product (0.24 g.), in the same manner, was carried out.

Anal.: Calcd. for $C_{16}H_{20}O_7(OCH_3)_2$: C, 55.94; H, 6.80; OCH_3, 16.0%; Mol. Wt., 387.

Calcd. for $C_{17}H_{22}O_8(OCH_3)_2$: C, 54.79; H, 6.79; OCH_3, 14.9%; Mol. Wt. 417.

Found: ash, 4.78, 5.05; C, 52.60, 52.61; H, 6.43, 6.41; OCH_3, 17.3, 17.5%.

Corrected for ash: C, 55.33, 55.34; H, 6.76, 6.74; OCH_3, 18.2, 18.4%.

Investigation of Fraction I - B

I - B was converted to a buff-colored, amorphous powder by the same method as was used for I - A.

Anal.: Found: ash, 0.0, 0.0; acetyl, 36.2, 35.9; nitrogen, 0.0, 0.0%; $[\alpha]_D^{25°}$ -18.1° (c = 1.496, methanol).

Conditions for the reacetylation duplicated those used for I - A, and 3.9 g. of I - B was recovered in 97.5% yield as a light cream-colored, amorphous solid.

Anal.: Calcd. for $C_{22}H_{20}O_{11}(C_2H_3O)_7(OCH_3)$: C, 58.41; H, 5.83; acetyl, 39.5; OCH_3, 4.07%; Mol. Wt. 761.

Calcd. for $C_{22}H_{22}O_{11}(C_2H_3O)_7(OCH_3)$: C, 58.25; H, 6.08; acetyl, 39.4; OCH_3, 4.06%; Mol. Wt., 763.

Found: C, 58.33, 58.25; H, 5.96, 5.95; acetyl, 39.2, 39.5; OCH_3, 3.12, 3.08%; Mol. Wt., 792, 750 (benzil as standard); $[\alpha]_D^{25°}$ -17.4° (c = 1.950, methanol).

Deacetylation was effected, as previously described, with 6 ml. of 0.425 M barium methoxide solution and a 6.24-g. sample of a mixture of reacetylated I - B and I -B in 65 ml. of anhydrous methanol. The observations made during the reaction and during the recovery of the product, 2.30 g. of a chocolate-brown amorphous solid, duplicated those for the deacetylation of I - A.

<u>Anal.</u>: <u>Found</u>: ash, 1.39 , 1.01%.

The acid hydrolysis of a 0.0490-g. sample of deacetylated I - B (corrected for ash) increased the copper-reducing power from 0.1015 g. as glucose to 0.440 g. as glucose, per g. of ash-free material, and fermentation reduced the latter value to 0.0826 g.

<u>Anal.</u>: <u>Calcd.</u> for the expected deacetylated monoglucoside:
Mol. Wt. 468.
<u>Found</u>: 1 mole "glucose" per Mol. Wt. of 534, 505.

The solution of 2.12 g. of deacetylated I - B in 30 ml. of anhydrous methanol seemed complete. Nevertheless, the liquid was filtered and the sample recovered in the usual manner, in 100% yield.

<u>Anal.</u>: <u>Calcd.</u> for $C_{16}H_{21}O_8(OCH_3)$: C, 54.83; H, 6.50
OCH_3, 8.34%; Mol. Wt., 372.

<u>Calcd.</u> for $C_{18}H_{23}O_9(OCH_3)$: C, 55.05; H, 6.35;
OCH$_3$, 7.48%; Mol. Wt., 415.
<u>Calcd.</u> for $C_{20}H_{25}O_{10}(OCH_3)$: C, 55.24; H, 6.20;
OCH$_3$, 6.79%; Mol. Wt., 457.

<u>Found</u>: ash, 0.78, 0.80; C, 54.45, 54.45; H, 6.19, 6.27;
OCH$_3$, 8.95, 8.96%.
Corrected for ash: C, 54.89, 54.89;
H, 6.24, 6.32; OCH$_3$, 9.04, 9.05%.

Hydrolysis of a sample (0.0605 g., corrected for ash) with emulsin failed to increase the original copper-reducing power from 0.125 g. of glucose per g. of ash-free material.

Methylation of 1.41 g. of the deacetylated material in methanol solution with 2 successive 1-g. amounts of diazomethane, added 24 hours apart, resulted in 1.30 g. of an amorphous solid.

<u>Anal.</u>: <u>Found</u>: ash, 0.87; OCH$_3$, 21.2, 21.3%.
Corrected for ash: OCH$_3$, 21.4, 21.5%.

This product was methylated with a third 1-g. portion of diazomethane. The recovery of the orange-brown, amorphous solid was 1.14 g.

<u>Anal.</u>: <u>Calcd.</u> for $C_{19}H_{23}O_7(OCH_3)_3$: C, 57.86; H, 7.07;
OCH$_3$, 20.4%; Mol. Wt., 457.
<u>Calcd.</u> for $C_{20}H_{22}O_7(OCH_3)_4$: C, 57.82; H, 6.88;
OCH$_3$, 24.9%; Mol. Wt., 499.

Found: Ash, 0.81, 0.93; C, 57.42, 57.29; H, 6.97, 6.94; OCH_3, 22.2, 22.2%.

Corrected for ash: C, 57.92, 57.79; H, 7.03, 7.00; OCH_3, 22.4, 22.4%.

Investigation of Fraction II - A

Fraction II - A was changed to a buff-colored, amorphous powder in the usual manner.

Anal.: Found: ash, 0.0, 0.0; acetyl, 32.5, 33.1; nitrogen 0.0, 0.0%; $[\alpha]_D^{25^\circ}$, -23.5° (c = 2.002, methanol).

Reacetylation of 4.4 g., as already described, resulted in the 100% recovery of a nearly-white, amorphous solid.

Anal.: Calcd. for $C_{19}H_{19}O_8(C_2H_3O)_6(OCH_3)$: C, 57.80; H, 6.08; acetyl, 38.8; OCH_3, 4.66%; Mol. Wt. 665.

Calcd. for $C_{19}H_{21}O_8(C_2H_3O)_6(OCH_3)$: C, 57.62; H, 6.36; acetyl, 38.6; OCH_3, 4.65%; Mol. Wt. 667.

Found: C, 57.50, 57.59; H, 6.27, 6.20; acetyl, 38.6, 38.8; OCH_3, 3.44, 3.43%; Mol. Wt. 667, 708 (benzil as standard); $[\alpha]_D^{25^\circ}$ -22.0° (c = 2.275, methanol).

Deacetylation of 8.74 g. of a mixture of fraction II - A and reacetylated II - A in 90 ml. of anhydrous methanol with 4 ml. of 0.425 M barium methoxide solution duplicated the conditions for the deacetylation for I - A, and similar observations were made. The yield of light-brown, amorphous powder was 2.82 g.

Anal.: Found: ash 6.02, 6.54%.

A sample of 0.0486 g., corrected for ash, was subjected to acid hydrolysis, and then fermentation. The former increased the copper-reducing power (per g. of ash-free material) from 0.0813 g. as glucose to 0.520 g., while the latter reduced it again to 0.0923 g. of glucose.

Anal.: Calcd. for the expected deacetylated monoglucoside:
Mol. Wt., 414.

Found: 1 mole "glucose" per Mol. Wt. of 411, 421.

An attempt was made to de-ash the deacetylated fraction II - A by using an acidic resin. A 0.505-g. sample was dissolved in 10 ml. of water and 50 mg. of regenerated Amberlite IR-120 was added. The mixture was stirred vigorously for 5 mins., filtered, and the filtrate concentrated to dryness *in vacuo*. The residue was converted from a dark brown syrup to a light-brown, amorphous powder by solution in 15 ml. of methanol, precipitation with 120 ml. of ether, filtration and extraction of the precipitate with pentane. The yield was 0.271 g., or 53.6%, with 8.97, 8.70% ash and the ethereal filtrate, on concentration to dryness, yielded no residue. Thus the resin had retained almost half of the sample.

The removal of 0.045 g. of insoluble material was effected by mixing 2.14 g. of deacetylated II - A with 30 ml. of anhydrous methanol, filtering, and employing the usual recovery.

Anal.: Calcd. for $C_{20}H_{27}O_{10}(OCH_3)$: C, 54.99; H, 6.61;
OCH$_3$, 6.76%; Mol. Wt., 459.
Calcd. for $C_{22}H_{31}O_{11}(OCH_3)$: C, 54.96; H, 6.83;
OCH$_3$, 6.17%; Mol. Wt., 503.
Found: ash, 5.96, 5.86; C, 51.87, 52.02; H, 6.34, 6.36;
OCH$_3$, 7.95, 7.94%.
Corrected for ash: C, 55.12, 55.30;
H, 6.74, 6.76; OCH$_3$, 8.44, 8.43%.

An increase in the copper-reducing power (per g. of ash-free material) of a 0.0605-g. sample (corrected for ash), from 0.101 g. as glucose to 0.197 g., was caused by treatment with emulsin, in 24 hours. An additional 48 hours' treatment with emulsin, of proven activity only succeeded in increasing the latter value to 0.206 g.

A solution of 1.32 g. of the deacetylated material in methanol was treated with 2 successive 1-g. amounts of diazomethane, added 24 hours apart. Recovery gave 1.15 g. of a light brown, amorphous solid.

Anal.: Found: ash, 5.99; OCH$_3$, 17.8, 18.0%.
Corrected for ash: OCH$_3$, 18.9, 19.1%.

A third methylation with 1 g. of diazomethane was carried out, which resulted in 0.85 g. of a light orange-brown, amorphous powder.

Anal.: Calcd. for $C_{19}H_{23}O_7(OCH_3)_3$: C, 57.86; H, 7.07; OCH_3, 20.4%; Mol. Wt., 457.

Calcd. for $C_{20}H_{22}O_7(OCH_3)_4$: C, 57.82; H, 6.88; OCH_3, 24.9%; Mol. Wt., 499.

Found: ash, 5.84, 6.14; C, 54.30, 54.29; H, 6.53, 6.50; OCH_3, 18.9, 19.3%.

Corrected for ash: C, 57.77, 57.75; H, 6.95, 6.92; OCH_3, 20.1, 20.5%.

SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

1. The water-soluble, non-dialysable fraction from white spruce bark, previously obtained by another worker, was analyzed to determine the extent of changes caused by enzymatic hydrolysis over an eighteen-month period of storage, and previous analyses were verified.

2. Dialysis of the originally non-dialysable fraction against water was carried out over 50 days, in a specially-constructed dialyzing apparatus. At least 47.5% of the solids were found to be dialyzable. Detailed sugar analyses showed that glucose (both free and combined) made up nearly half (46.2%) of the dialyzable materials. The sugar was removed by fermentation with yeast.

3. Tannins were removed by large-scale adsorption on hide powder, and for the first time, the non-tannin constituents were made available for investigation.

4. A new technique was devised for the separation of the non-tannins, consisting of acetylation with acetic anhydride and pyridine to near-white, amorphous solids, which were subsequently adsorbed on, and fractionally recovered from, an alumina column. Detailed analyses of the fractions obtained threw some light on their chemical constitutions.

5. Systematic analysis of these acetates showed each to be a mixture containing components of varying methoxyl content, and of average molecular weight in the range of 625 to 770. Deacetylation recovered only 45% to 59% of the expected yield, and analytical results were not in accord with values calculated from the acetates. Each product, however, contained close to the calculated amount of glucose as a glucoside, which was sometimes stable, and sometimes unstable, to the enzyme emulsin. Methylation with diazomethane revealed the presence of one or two phenolic hydroxyl groups in the glucoside fractions.

6. Although the corresponding aglycones were not studied in detail, they were inferred to be partly methylated phenolic compounds of unexpectedly low molecular complexity, and having formulae in the range $C_{12}H_{12}(OH)_4$ to $C_{16}H_{15}(OH)_5$. Unlike crystalline glycosides of this general pattern, the above aglycones and their glycosides were present as major, rather than minor, constituents of the bark.

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