A STUDY OF THE ALKALINE EXTRACT OF WHITE SPRUCE BARK PRETREATED WITH LIQUID AMMONIA

by

John Raymond George Bryce

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GENERAL INTRODUCTION

It is very desirable to find good methods of utilizing the vast amount of bark which is produced as a by-product in the pulp and paper and lumbering industries. Up to the present, the attempts at making use of bark have been centered for the most part around burning it or using its fibrous fraction as such. However, more attention has been turned recently to the idea of using bark by separating it chemically into fractions which might be of value. For example, the bark of certain trees contains a significant proportion of tanning agents, but these do not occur in quantities sufficient to warrant commercial utilization; if other useful products could be isolated from these barks, extraction of the tannins might also become feasible. The first step in such an approach to the bark problem would appear to be a study of the chemical structure of bark constituents.

In the last few years, work has been conducted in this department on the chemical nature of white spruce bark. The substances extracted by alcohol and by water were studied by conventional methods, but an original approach to the study of the extractive-free residue was used. Instead of the usual extraction with sodium hydroxide, the bark was treated with liquid ammonia. This process made available two additional fractions - one soluble in liquid ammonia and another made water-soluble by the liquid ammonia treatment.

The above extractions left about 48% by weight of the original bark as a dark brown mass. The research now to be reported deals with the separation and classification of the substances extracted from this residue by aqueous sodium hydroxide, and pays particular attention to the polysaccharides found in the extract.

HISTORICAL INTRODUCTION

Probably the greatest single waste product of our forests today is bark. The Canada Year Book for 1952-53 (1) has reported that 12,497,926 cords of unpeeled wood were consumed in Canada in 1950 by the pulp and paper industry. When Richter's estimate (2) that bark makes up 9 to 15% of the tree by volume is accepted, it follows that well over 100,000,000 cu.ft. of bark are produced by the Canadian pulp and paper industry alone. The production of the lumbering industry is doubtless just as large.

The disposal of this quantity of bark is one of the major problems of the log-consuming industries today. There was a time when bark could be discharged into rivers and streams, but in view of the expansion of industry and the spread of population in recent years, legislation now tends to prohibit such pollution. The uses to which bark is now being put can be broken down into three classifications - burning, use of whole bark fibres, and use of bark after separation into fractions.

The most common utilization of bark at the present time is undoubtedly burning to produce heat. However, in most mills, bark arrives in a spongy, water-logged condition. Lebaron (3) has shown that bark containing more than 80% of water will not support combustion. In consequence, expensive pressing equipment must be installed, and even then the moisture is only reduced to 65%. A large part of the heat produced

in the combustion will therefore be consumed in evaporating this residual water, so that the operation becomes merely a method of disposal rather than a practical utilization of the bark. Vroom (4) has studied the pyrolysis of bark in a fluidized bed at different temperatures and using a variety of fluidizing gases. This process yields three fractions - an organic distillate, a gaseous portion, and a pyrophoric charcoal. The first portion might be utilized chemically, while the latter two might be burnt with only a little less heat output than that which would be obtained from the original wet bark.

There have been many suggestions involving the utilization of bark fibres. These include, for example, the manufacturing of insulating boards (5), fortification with nitrogen for use in building up soils (6), and as a material to be impregnated with tars and asphalts (7). Chemical pulping of bark results in low yields, and a high consumption of chemicals (8) (9); the resultant product is only a low grade pulp, although its use as newsprint wrappings has been suggested.

The separation from bark of fractions, or even individual compounds, of commercial value might result in a more lucrative utilization of bark wastes. As an example of such separations, the recent leasing of patents (10) for the extraction of tannins, waxes, and dihydroquercitin from Douglas fir bark may be cited. The most valuable constituents extracted from bark at present are probably the tannins. It

has been pointed out (11) that Canada imports 99.5% of its tannin requirements. Many of the problems concerning the extraction of bark for tannin are considered in a recent bulletin published by the Northeastern Wood Utilization Council (12). The feasibility of extracting tannins differs widely from one species of tree to another. Thus, Kurth and Chan (13) have reported that extraction from Douglas fir bark would be practical, whereas Beebe, Luvisi, and Happisch (14) thought that Tennessee Valley oak bark would not be a practical source. Kurth (15) has observed, however, that the tannin content of bark is considerably lowered by transporting or storing the logs in water, a common practice at present. Perhaps chemical debarking, which would permit the bark to be removed and extracted in the woods, might be useful in this respect. This subject was recently reviewed by McIntosh (16).

Since a chemical separation of the bark now appears to be the most interesting channel of utilization, considerably more knowledge of the structure of bark constituents should be gained, and the methods of isolating the useful components should be investigated. In most previous work, the emphasis has been on separating bark into various broad groups of substances by means of classifications of differing solubilities in various solvents and reagents. Little has been done to study the chemical nature of the fractions in detail. The results of this work have been discussed in review articles by Segall and Purves (17) and by Kurth (18).

In industrial terminology "bark" refers to that portion of the log which can be readily removed by peeling. Botanically, bark is more precisely defined as all the tissues external to the cambium (17). Prior to extraction, the bark has often been separated into "inner" and "outer" bark, which are considered to correspond to the "living" and "non-living" portions respectively. Pigman and Anderson (19) have supported this view experimentally by showing that certain constituents, such as protein, sucrose, and pectin, which are usually associated with living tissue, are much more plentiful in the inner than in the outer bark. Jensen (20) has studied the relationship between the chemical composition and the anatomical structure of outer white birch bark by examining bark sections microscopically before and after extraction with certain solvents. He suggests that the ether-soluble portion arises from the layers formed in spring, while the components soluble in alcohol or sodium bisulphite are concentrated largely in the darker "summer outer bark".

The method most commonly used for obtaining and separating the extractable components of bark was first worked out by Zellner and his collaborators. This method involves exhaustive extraction of the bark, first with alcohol and then with water, and then sub-division of the alcohol-soluble fraction into water-, petroleum ether-, ethyl ether-, and alcohol-soluble fractions. The details of this method, the types of compounds appearing in each fraction, and the results

obtained by Zellner and his associates in applying this technique to various European hardwood barks have been thoroughly summarized in the above-mentioned review articles. Kurth and Hubbard (21), in a study of Ponderosa pine bark, have attempted to obtain a better fractionation by the direct extraction of the bark with benzene, ethyl ether, alcohol, and water in succession, but this sequence would involve considerable complication in large-scale operations. The initial work carried out in this laboratory by Harwood (22)(23)(24) and Bishop (24)(25) on white spruce bark followed the method of Zellner. This work is summarized in Figures I and II.

Less knowledge is available on the constituents of the extractive-free bark than on the extractives. For this reason, it is still uncertain whether the common structural elements of wood, such as lignin and cellulose, occur as such in the bark. Many of the investigations have been conducted on material which has been isolated under conditions of such vigour that considerable structural change is likely to result. For example, the extractive-free bark is usually re-extracted with sodium hydroxide solutions, at temperatures well over 100°C. Another reason why the early researches on extractive-free bark are of limited value is because of the use of inadequate analytical methods. It has been customary to apply the analytical methods used for woods, but more recently the applicability of these to bark has been questioned, and much of the earlier data have become meaningless. Kurth (26) has

FIGURE I

Fractionation of the Concentrated Methanol Extract (22)

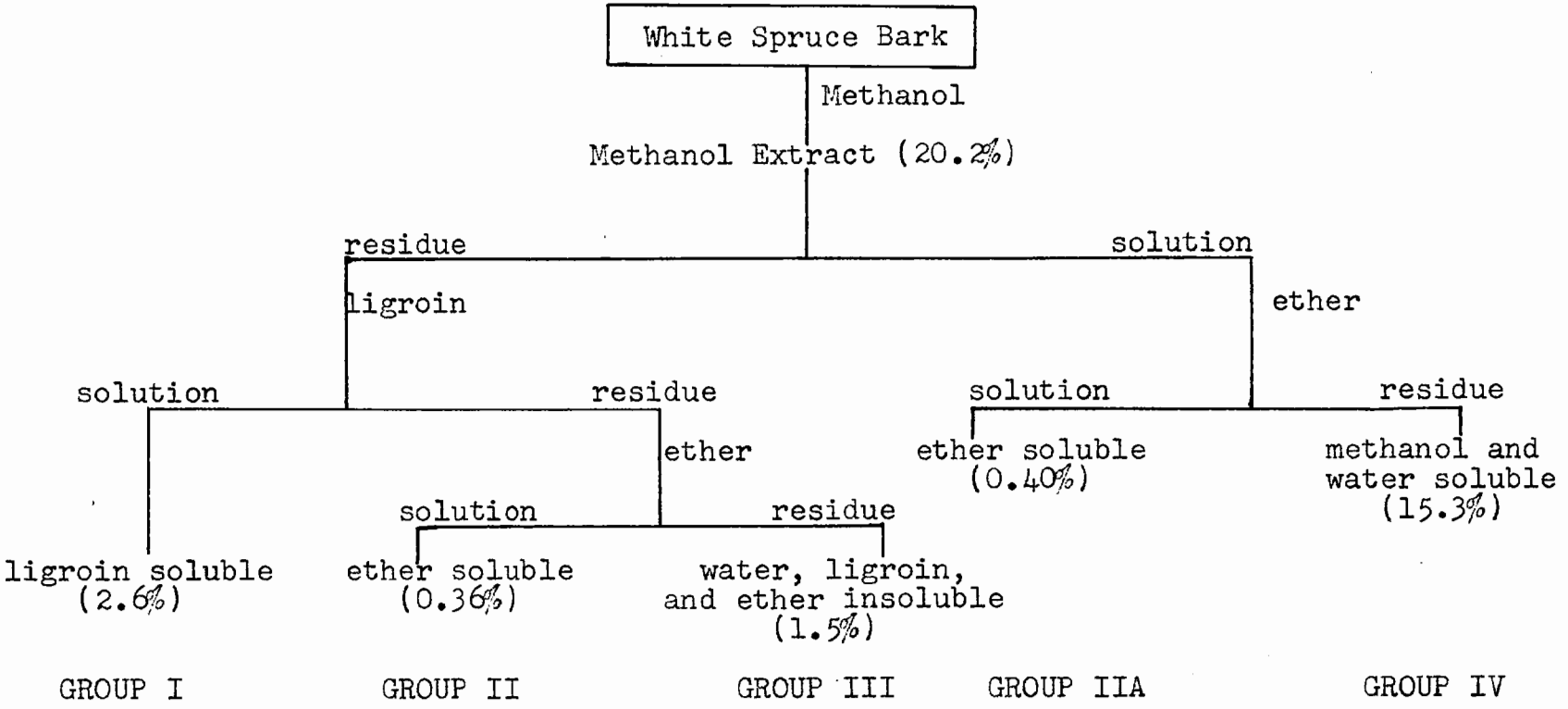
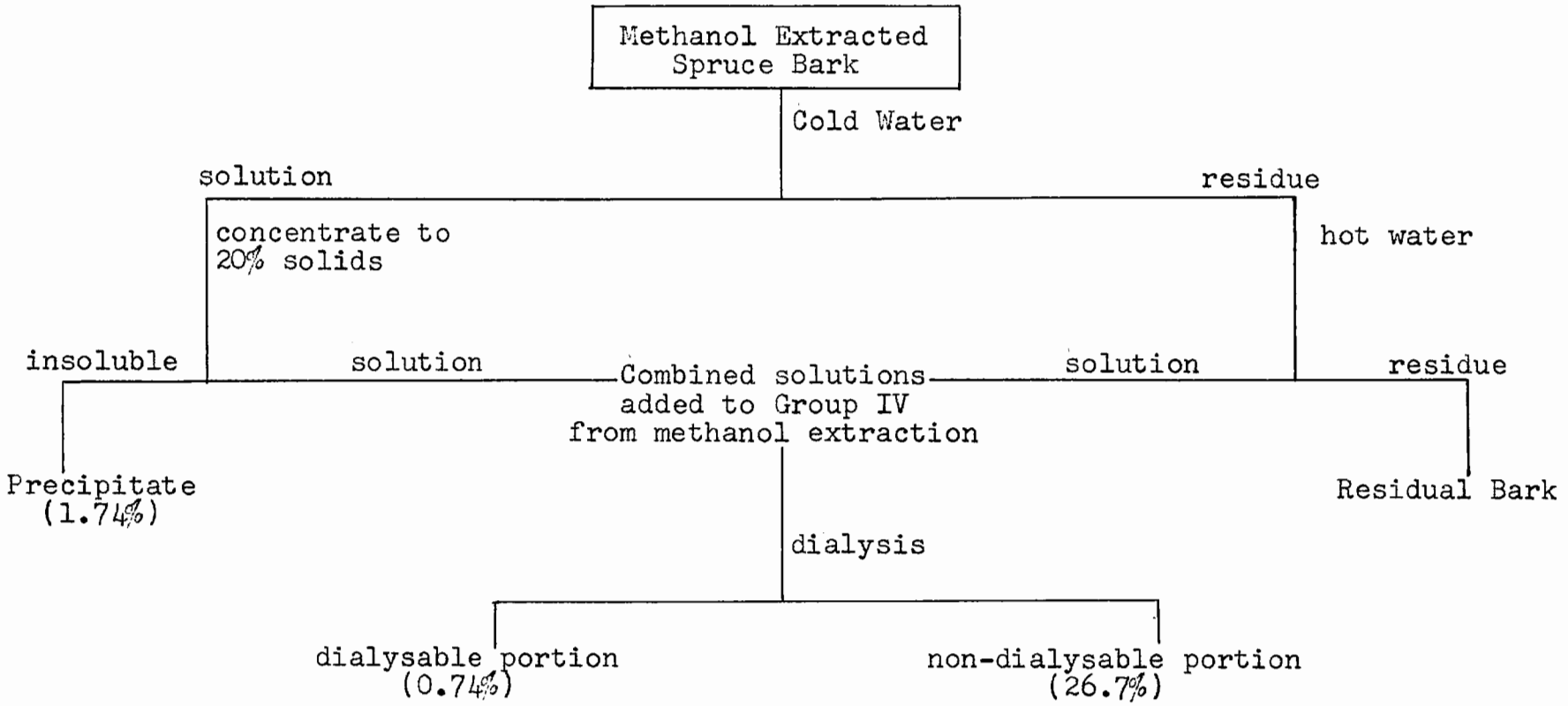


FIGURE II

Fractionation of the Aqueous Extract (25)



shown that neither the Cross and Bevan nor the ethanolamine determinations of cellulose give complete delignification in the case of bark. Many workers have pointed out that suberin, tannin, phlobaphenes, and other non-lignin bark constituents are insoluble in 72% sulphuric acid, and that in consequence the Klason lignin determination is not a true measure of the lignin content. Hilpert and Knackstedt (27) have suggested that the usual method of determining pentosans may lead to low results with bark, because of the condensation of the furfural formed from the pentosan with the phenolic substances present. In the subsequent discussion, the extractive-free bark will be dealt with under three structural subdivisions: suberin, bark "lignin" and polysaccharides.

Suberin, the major constituent of the cork cells of bark, was isolated by Zetsche and co-workers (28) by boiling extractive-free bark with 4% sodium sulphite solution. They later showed (29) that the saponification of suberin yielded phloionic acid, $HOOC-(CH_2)_7-(CHOH)_2-(CH_2)_7-COOH$, phellonic acid, $HO-(CH_2)_{21}-COOH$, and n-eicosanic acid, $CH_3-(CH_2)_{18}-COOH$. More recently, Jensen studied the structure of suberins, and isolated eicosanedicarboxylic acid, $HOOC-(CH_2)_{20}-COOH$, phellonic acid (30), phloionic acid (31), an unidentified C_{18} acid (32), and phloionolic acid, $HOCH_2-(CH_2)_7-CHOH-CHOH-(CH_2)_7-COOH$ (33). Jensen also proved the chemical structures given for these acids. He concluded that suberin was a polyestolide of hydroxy fatty acids. Hergert and Kurth (34) separated the cork

cells from the bast fibre of Douglas fir bark by a grinding and sieving process. They found evidence of a linkage between the hydroxy acids and a phenolic acid which they had isolated from an alkaline extraction of the bark, and concluded that, in Douglas fir cork, "the hydroxy acids are not only esterified to the phenolic acid, but also to each other in an etholide type linkage". They did not attempt to explain the function of the glycerol which they also obtained in the alkaline extract. Jablonski (35) seems to have uncovered a new approach to the study of suberins, when he extracted white spruce bark with liquid ammonia. He was able to show the presence in the extract of fatty acid amides, as well as glycerol, and he suggested that the glycerol might act as a cross-linking agent, contributing to the elasticity and chemical inertness of suberin.

It has long been a matter of controversy whether or not any "lignin" is present in bark. The study of this "bark lignin" is complicated because there is such a wide variety of phenolic compounds present in the bark, and because the nature of these compounds seems to vary considerably from one species of tree to another. The difference between wood and bark lignins is shown in the lower methoxyl content of the latter, and the lower yield of vanillin that is produced on oxidation with alkali and nitrobenzene. The procedures used in the isolation of bark "lignin" have often been rather drastic, since bark "lignin" appears to be bound to the carbohydrate

more strongly than wood lignin, and in many cases conditions which would decompose sensitive structures have been used. As mentioned above, the Klason lignin determination cannot be considered satisfactory for bark. For this reason, the term "bark lignin" is very indefinite, since there are no reliable methods of determining or isolating it.

The work on bark "lignin" up to 1945 has been carefully reviewed by Segall and Purves (17), and can be treated in less detail here. Wacek and Schön (36) have shown that the yield of Klason lignin from outer spruce bark varies greatly with the extent to which the extractives have been removed, and suggest that this is largely due to the interference of the tannins present. Sharkov (37) confirmed this view by showing that 72% sulphuric acid produces insoluble residues from fir bark tannin, and that the residues from the hot water and alcohol extracts of fir bark have similar carbon and hydrogen values. He concluded that these "lignins" are merely the insoluble products that natural tannins give with acid.

Clotofski and Junge (38) found that the aqueous extract from beech bark yielded 6% vanillin on oxidation with alkaline nitrobenzene, and this result indicated the presence of methoxylated substances resembling wood lignin. They also applied several of the methods worked out for wood lignin to their bark and found that Freudenberg's "cuprammonium" procedure gave the lowest yield of "lignin" (18%), but that this residue had the highest methoxyl content (15.5%) and gave the

best yield of vanillin (16.7%) on oxidation. Hilpert and Knackstedt (27) studied and compared the yield of 72% sulphuric acid "lignin" and its methoxyl content for five European softwoods and also for hardwood bark fibre fractions. The yields of "lignin" ranged from 6.2% for spruce to 33.7% for eucalyptus, and the "lignin" methoxyl values from 1.4% for pine to 14.6% for maple. The wide divergency of these values led the authors to conclude that their products were mere artifacts. This viewpoint is now thought to be too severe, and while bark "lignin" is probably not structurally identical with wood lignin, there is a bark cell wall component functionally analogous to lignin in wood.

Lehmann and Wilke (39) treated solvent-extracted pine bark with 3% caustic soda at 130°C., and obtained "alkali-soluble phlobaphenes". They noted that the treatment of these soluble extracts with a hydrochloric-sulphuric acid mixture converted them to "alkali-insoluble lignins" which had an increased carbon content. They also found that as the ease of the extraction decreased, the methoxyl content of the extract increased. This observation confirmed an earlier one by Sharkov and Kalnina (40). Lewis, Brauns, Buchanan and Kurth (9) (41) isolated three lignin fractions from solvent-extracted redwood bark fibre. The first of these (lignin A) was extracted by a cook with 12.5% alkali at 160°C., and was characterized by its low methoxyl content (2.7%). The second portion (lignin B) was obtained by repulping the residue with

22% alkali at 160°C., and had a higher methoxyl content (11.6%). On the basis of methylations with dimethyl sulphate and diazomethane, and of acetylation, it was concluded that lignin A contained a considerable number of free phenolic hydroxyl groups, and a significant number of carboxyl groups. The presence of alcoholic hydroxyl groups was also indicated. Lignin B possessed some carboxyl units, but Lewis and his colleagues could not ascertain whether these were really a part of this fraction or were due to contamination by lignin A. Lignin B had fewer free phenolic hydroxyl and more alcoholic hydroxyl groups than lignin A. In general, lignin B was much more similar to wood lignin. Lignin C was very resistant to pulping, and could not be extracted by sodium hydroxide or sulphite, but was removed as the phenol derivative, by heating the bark residue with phenol. This derivative contained 11.1% of methoxyl groups. Even the phenol left a small amount (2.7%) of the Klason "lignin" unextracted. The bark "dust" fraction was found to consist to a large extent of phenolic carboxylic acids very closely resembling lignin A.

Kurth and his co-workers then discovered similar lignin-like constituents in Douglas fir bark. Kiefer and Kurth (42), examining the extractive-free bast fibres, found that about half of the Klason "lignin" content was removed in 1 hour at 100°C. by 1% sodium hydroxide. The extracted material, a phenolic acid somewhat similar to that extracted from redwood bark, contained only 4.3% of methoxyl groups, and the presence

of carboxyl groups was shown both by the methylation method and by infrared spectroscopy. Another "lignin" fraction, with 14.3% of methoxyl, was extracted with dioxane containing 0.4% of hydrogen chloride, while the residual lignin, containing 11.1% of methoxyl, was obtained by the 72% sulphuric acid method. Hergert and Kurth (33) heated the extractive-free cork fraction from Douglas fir bark with boiling 1.2N alcoholic potassium hydroxide, and removed a substance containing 4.34% of methoxyl and 4.7% of carboxyl groups. The ultra-violet absorption spectra showed this phenolic acid to be more similar to bark phlobaphenes than to wood lignins.

Gleason (43) treated methanol- and water-extracted spruce bark with 5% sodium hydroxide at room temperature, and obtained 14% of the original bark as a precipitate on acidification of the alkaline extract. This precipitate had 1.45% of methoxyl groups. He studied the oxidation of this bark "lignin" with alkali and nitrobenzene but was unable to isolate any vanillin. After complete methylation with dimethyl sulphate, however, the bark yielded 0.05% of vanillin on oxidation. Gleason also submitted his extract to high pressure hydrogenation in dioxane over copper chromite at 200°C. and 3000 pounds pressure. From the product, he obtained small yields of dimethylamine, 4-methylcyclohexanol-1, and 4-n-propylcyclohexanol-1. In view of these results, he concluded that bark "lignin" resembled soil humic acids more closely than wood lignin.

The existence of true cellulose in bark seems to be in

as much doubt as the existence of true lignin. Determination of the extractives, ash, pentosans, and Klason lignin usually leaves unaccounted-for about 22-30% of the original bark. The attempts to elucidate the nature of this material, which should include the cellulose, have given quite contradictory results. Thus, Lehmann and Eisenhut (44), investigating the insoluble residue remaining after pulping pine bark with 10% sodium sulphite at 140 to 150°C., claimed that the hydrolysate of the residue contained much galactose, although the identification was only by the mixed melting point or decomposition point of the osazone, which was not a reliable method. Frieze, Clotofski and Doderlein (45) obtained a similar result when they isolated galactose, rhamnose, and xylose, but no glucose, after degrading alkali-pulped pine bark with an acetic anhydride-sulphuric acid reagent. Hilpert and Knackstedt (27) went so far as to suggest that the cellulose they isolated by cooking various barks with 5% caustic soda was a mere artifact. There seems to be little evidence to substantiate so drastic a conclusion, however.

On the other hand, Wacek and Schön (36), using pine bark pulped with alcoholic nitric acid, obtained a product which still had a significant amount of pentosan and of methoxyl groups, but which gave principally glucose on hydrolysis. Lehmann and Wilke (39) pulped extractive-free bark with caustic soda, and then digested the residue with alcoholic nitric acid, alkaline hydrogen peroxide, or chlorine dioxide.

The residual material still contained significant amounts of pentosan, gave an X-ray powder diagram not identical with that of cellulose, and was unusually resistant to hydrolysis with 43% hydrochloric acid. Clotofski, Weikert and Nick (46) obtained a "skeletal substance" by pulping extractive-free bark with sodium sulphite. They showed that the hydrolysate from this pulp contained considerable amounts of fermentable sugars, but could not isolate any pure glucose, galactose or mannose from it, possibly because of interference by the phenolic material that was also present.

A significant contribution to this work on bark cellulose was made when Lewis and his collaborators (9) investigated solvent-extracted redwood bark fibres. The product from an alkali pulp still contained 30% of lignin. In this case, however, the Cross and Bevan procedure appeared to yield a lignin-free product, which contained 89.6% of α-cellulose. They found the unpulped, extractive-free fibre to contain 46.9% of glucose, with lesser amounts of mannose and xylose, and they concluded that the redwood bark fibre was inherently a cellulose fibre, but that the cellulose was more strongly linked to "lignin" than was wood cellulose. Kurth and his associates (21) (34) (47) (48) (49), working with various Western pine, cedar and fir barks, nevertheless found that delignification by either the ethanolamine or the Cross and Bevan procedure was quite unsatisfactory. They preferred the sodium chlorite treatment of Wise (50), but even this procedure

led to degradation and loss in carbohydrate material when the lignin content was reduced below 6%. Since the predominant sugar in the holocellulose hydrolysate was glucose in each case, they concluded that the fibre was cellulosic in nature.

Of the non-cellulosic carbohydrates in bark, pectin, gums, mucilages, hemicelluloses and starch have all been suggested as possible constituents, but very little investigation of these substances has taken place, and these claims are seldom backed up by the actual isolation of the material. One of the rare cases in which the structure of a polysaccharide isolated from bark has been studied was reported by Gill, Hirst and Jones (51) (52), for a mucilage which had originally been isolated by Anderson (53) by the aqueous extraction of the bark of the slippery elm. They showed this mucilage to be composed of D-galactose, L-rhamnose, and D-galacturonic acid residues, and proposed a tentative structure on the basis of the products from the hydrolysis of the purified methylated mucilage.

Sanderson (54) subjected extractive-free white spruce bark, which had been pretreated with liquid ammonia, to another extraction with water, and obtained carbohydrate and pectic materials, together with tannins and other non-carbohydrate contaminants. After bleaching his samples with acidulated sodium chlorite solution he was able to show that the polysaccharides were based on galactose, glucose, xylose, arabinose, and uronic acids, but, even after considerable fract-

ionation, he was unable to isolate a pure material for detailed structural study. About the same time, Jablonski (35), working with the liquid ammonia extract, demonstrated the presence of carbohydrate material, which when fractionated yielded an araban and a glucosan, but he did not investigate these crude products in detail.

The interesting group of polysaccharides called the pectins are widely distributed throughout the plant kingdom. The main constituent of this group is a polygalacturonic acid, or pectic acid, although some of the carboxyl groups usually occur as the methyl ester. The free carboxyl groups are often involved as salts, calcium and magnesium being the two most common cations concerned. Associated with this pectic acid, however, are a galactan and an araban. Since these three substances almost always occur together, and since their solubilities are so similar, they are always extracted together, and so all three are classified as the pectic substances. Hirst and Jones (55) and Whistler and Smart (56) have contributed excellent reviews on the chemistry of these materials, while Kertesz (57) in a recent book has covered the principal aspects of this field. Nevertheless, reviews on pectic materials in wood seem to be lacking.

The pectic substances occur most abundantly in fruits, roots and young growing tissue, and it was at one time believed that they did not occur in mature plant tissues. However, their presence has now been shown in mature cotton, wood, bark and

grain kernels in low concentrations. The physiological role of pectin in plants is not certain. Their most important function appears to be as a cement to hold the cells together, although other substances, such as lignin, serve this purpose as well. Because of their hydrophilic nature, pectins can hold several times their own weight in water, and they probably also play an important part in the ability of plant tissue to take up and hold water.

It was at first believed that pectin was formed early in the growth of the plant and later became converted into lignin, mainly because von Fellenberg (58) found unlignified tissue contained a much higher concentration of pectin than lignified tissue. Fuchs (59) suggested that hemicelluloses were an intermediate in this transformation. This view was disproved by Buston (60) who showed that the total pectin content of rosewood during lignification did not decrease, although the absolute concentration fell because of the abundant formation of cellulose in the secondary wall, and of the formation of lignin and hemicelluloses. The existence of a chemical linkage between pectic acid and other polysaccharides in plants has also been discussed. Sucharipa (61) suggested an ester linkage between pectin and cellulose, while Henglein (62) favoured intermolecular calcium salt bridges between pectic acid and free carboxyl groups in cellulose molecules. Neither of these ideas was substantiated by sufficient experimental evidence. For a long time, however, there

was much controversy as to whether the araban and galactan were chemically linked to the pectic acid. Nanji, Paton and Ling (63) proposed a cyclic pectin structure containing galacturonic acid, galactose, and arabinose, but Ehrlich and Schubert (64) found that pure araban could be extracted from the mixture by autoclaving in water. Schneider and Bock (65) believed that the three components occurred in separate polysaccharides, although they had to use acid conditions to obtain pectic acid free from the other two. Hirst and Jones (66) (67) then succeeded in separating some pure araban and galactan from selected crude pectic extracts by mild physical separations, although neither was completely removed by these methods. This result was considered to indicate that there was no linkage between the araban and galactan. Speiser, Eddy and Hills (68) took the opposite viewpoint, and measured an activation energy of 18.5 kcal. for the acid cleavage of the proposed ester linkage between pectic acid and the "ballast materials" (araban and galactan).

Hirst, Jones and their collaborators showed the araban to be a highly branched polymer of 1,5-α - linked arabofuranose residues, and the galactan to consist of linear anhydrogalactopyranose units linked in the β configuration through the 4 position. The evidence for these structures is given by these workers in their review article (55). The early workers were able to identify galacturonic acid as the main product of the hydrolysis of pectic acid, but since they were not able to

obtain pure material, made little progress on the structure. Baur and Link (69) obtained a pure but degraded pectic acid by heating crude pectin with methanolic hydrogen chloride. The galactan and araban were preferentially hydrolysed by this treatment, leaving polygalacturonides of eight to ten uronic acid residues. Schneider and Bock (70), by measuring the viscosity of dinitropectin, found undegraded pectin had an average degree of polymerization of about 700. Henglein and Schneider (71) studied the physical properties of this nitropectin in solution, and suggested a 1,4-glycosidic linkage as the basis of the pectic acid structure. The first reliable chemical evidence of this structure was put forward by Levene and Kreider (72), who oxidized a pectic acid with periodic acid, and, after hydrolysis of the product and oxidation with bromine, obtained D-tartaric acid, indicating that free hydroxyl groups occurred on the C_2 and C_3 atoms. Since the extreme resistance of pectic acid to acid suggested the more stable pyranose ring structure, the C_4 position was left for the glycosidic linkage. The highly dextrorotatory optical rotation suggested an α-glycosidic configuration. Hirst (73), Beaven and Jones (74), and Smith and Luckett (75), have confirmed much of this structure by methylation studies of various degraded pectic acids, but since no trimethylgalacturonic acid has yet been isolated from the non-reducing end group, the position of the linkage has not been established with absolute certainty. However, recently Jones and Reid (76)

have shown the presence of the 1,4 linkage in a trigalacturonide isolated by enzymatic hydrolysis.

This structural work disproved another idea favoured by the earlier workers. Candlin and Schryver (77), noting the configurational similarity between D-galactose, D-galacturonic acid, and L-arabinose, suggested that pectic acid was formed by oxidation of the primary hydroxyl group of the galactan and that the araban was then formed by decarboxylation. Since, however, the araban was found by Hirst and Jones to be based on furan and not pyran units, the above mechanism would have to include, in addition, hydrolysis and repolymerization steps.

Earlier work on the occurrence of pectin in woods includes that of Ritter (78), who conducted a microchemical investigation of the middle lamella using solvents for pectin and lignin. He found a considerable amount of lignin (70%) but no evidence for pectin, and concluded that in woods, lignin replaced the pectin of softer plant tissues as the bonding material between the cells. According to Kerr and Bailey (79) (80), however, in completely delignified wood the cells did not separate until they were treated with pectin solvents. They concluded that pectic materials also played a role in the binding of the cells. Bailey (80) also showed that while the major portion of the supposed pectin was located in the middle lamella, some was also present in the primary cell wall in lower concentration.

Pectin was conclusively proved to be present in woods in 1925, when Miss O'Dwyer (81) succeeded in isolating the substance from beechwood. She treated the wood meal, which had been previously extracted with water and 4% caustic soda, with hot aqueous ammonium oxalate. After the removal of tannins from this extract with hide powder, pectin amounting to 0.3-0.5% of the original wood was precipitated on acidification, and was characterized by optical rotation and by the yield of furfural. Another early isolation of pectin from woods was by Preece (82) who showed boxwood to contain 0.4% of pectin by extraction with hot ammonium oxalate solution.

The most thorough study of pectins in wood was that of Anderson and his co-workers, who showed that, while pectin occurred in somewhat lower quantity in woods than in other plants, it was present in all the wood species studied. These species included black locust (83), lemonwood, mesquite, and white pine (84), aspen (85), cottonwood (86), Douglas fir, western hemlock, Western red cedar, and black spruce (87). The yield of pectic material was less than 1% in all cases. These workers isolated the wood pectic substances in three fractions - pectin A by extraction of the extractive-free wood meal with 0.05N hydrochloric acid, and pectin B by treating the residue with 5% ammonium hydroxide. A further portion, pectin C, was resistant to this treatment, and could only be isolated by first chlorinating the residue and extracting it with ethanol before the main extraction with ammonium hydroxide.

Each of these fractions, A, B and C, after purification by repeated precipitations as the calcium salt, yielded a pure pectic acid quite similar to that isolated from fruits.

Pectins have been reported to occur in many other woods by various workers who, however, in general, do not characterize their material in the careful manner that is shown in the work of Anderson. The presence of pectic material is often based on a uronic acid determination of the wood. Claims such as these are of little significance, since uronic acid residues also occur in other structures commonly found in wood.

While a few workers have suggested that pectin is present in bark, information as to its amount is non-existent, and the isolation and characterization of a pure sample is rare. Graham and Rose (88) reported that "pectinous matter" interfered with extraction of tannins from balsam bark by hot water, but gave no details as to the nature of this material. Heiduschka and Chang (89) claimed that the bark of the paper mulberry contained 9.1% of pectin, but this was only based on the amount of methanol released by saponification in alkali. This method is of little value when applied to bark, since the nature of many of the constituents is unknown. Buston and Hopf (90), working with ash bark, and Haas (91) who studied the bark of several citrus fruit trees, isolated the pectic material by precipitation of the calcium salt, but did no further characterization. Hay and Lewis (92),

investigating balsam bark, suggested that the gelatinous material which covered certain cells after the solvent—extracted bark was heated in water was pectin-like in nature, but they did not isolate the material.

Probably the best example of the isolation of a pectic acid from bark was reported by Lewis, Brauns, Buchanan and Kurth (9) in their study of redwood bark. These workers found that the bark "dust" fraction yielded 9.6% of an aqueous extract which contained 58.3% of uronic anhydride and yielded mucic acid on oxidation with 25% nitric acid. After oxidation with bromine water and precipitation with alcohol, a product of 76% uronic anhydride content and specific rotation +233° was obtained. These figures are comparable with those of pectins from other sources.

Black spruce bark has also been shown to contain a considerable amount of pectic material. Anderson and Pigman (19) found the inner bark of this tree to contain 10% of uronic anhydride, which they assumed to be pectic in nature. In a later report, Pigman, Anderson and Leaf (93) isolated some of this material by extraction of the bark holocellulose with 5% ammonium hydroxide, but the yield was not reported. The specific rotation of this material was only +80°, and the uronic acid content only 65%. Only 28% of the theoretical yield of galacturonic acid was isolated on enzymatic hydrolysis of the sample, which appeared to be an impure pectic acid. Sanderson (54), working on extractive-free white spruce

bark, which had been treated with liquid ammonia, isolated a fraction by aqueous extraction which was found to contain pectic material amounting to 1.9% of the original bark. This pectin was characterized by oxidation-hydrolysis to mucic acid, using a mixture of bromine and hydrobromic acid.

As the main reserve material in plants, starch occurs widely throughout the plant kingdom, and its presence in woods and barks would not be surprising. Several workers inferred its presence on the basis of colourations with iodine, but other materials, such as hemicelluloses, sometimes give this test and a positive identification of starch can only be made after isolation. The first definite isolation of starch from a wood was reported in 1935 by Campbell (94), who found that the tannins could be removed by extraction with water at 60°, and that quite pure starch was then extracted with water at 100°. By this means, he extracted 1.38% of starch from oak sapwood, 0.43% from walnut sapwood, but only a little from the heartwoods. This starch was characterized by its optical rotation and by its fermentation by the enzyme taka-diastase. O'Dwyer (95) later found that the glucosan she had observed in her hemicellulose extracts was hydrolysed by the same enzyme, and was, therefore, apparently starch.

About the same time, Niemann, Roberts and Link (96) isolated a starch polysaccharide from the woody tissue of the apple tree by extraction with hot 20% aqueous ethanol. The material was identified by its rotation and viscosity, and

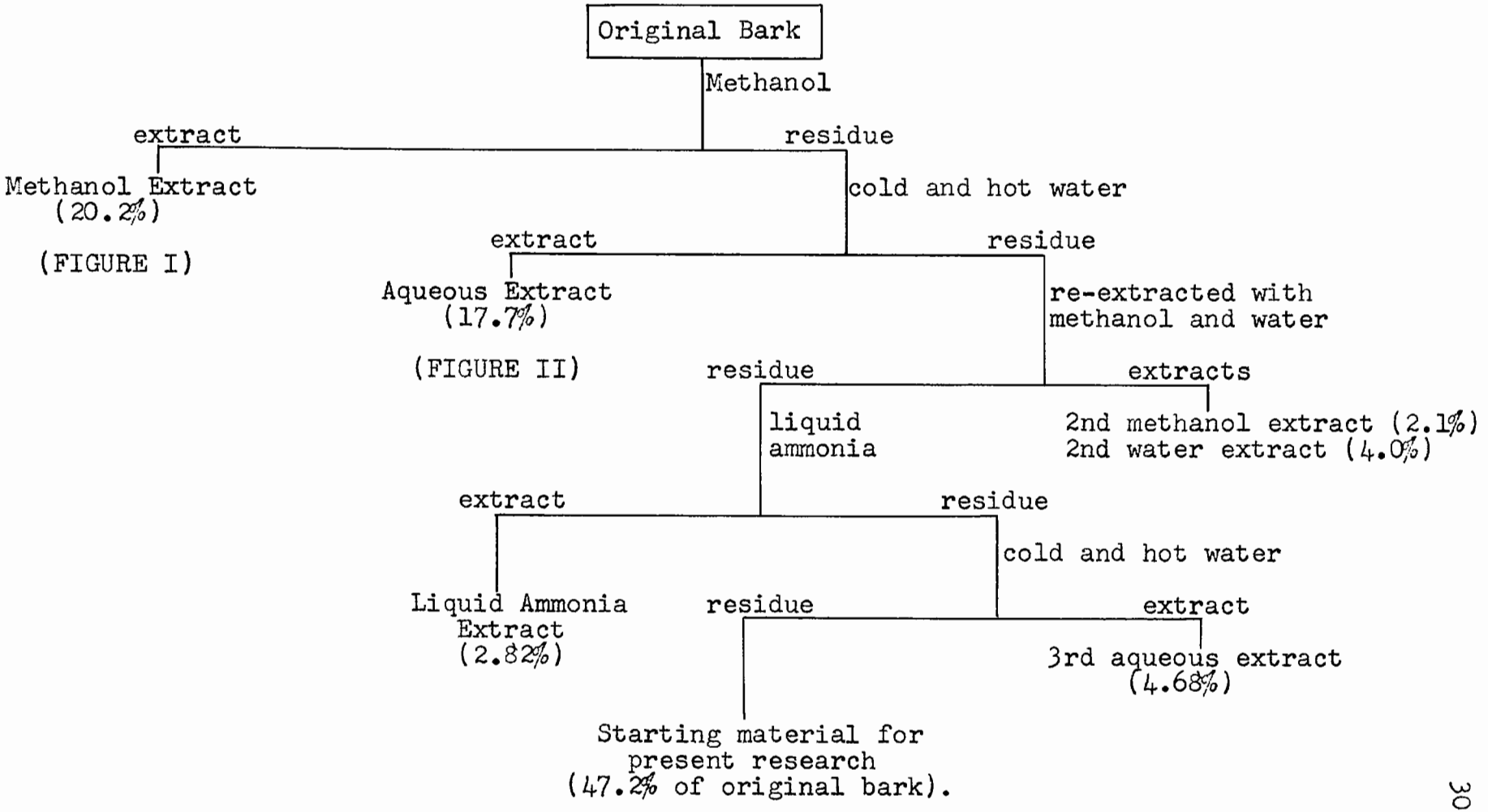
by the rotation of its triacetate. The products from hydrolysis with acid and enzymes, and from methanolysis, were studied, and each contained glucose as the only sugar unit present. Spoehr and Milner (97) isolated a starch from the wood of black locust roots by extraction with hot water. A more detailed study of the starches from elm and maple has been reported by Campbell, Percival and their associates (98) (99). This work has shown wood starch to be quite similar to starch from other sources. They showed it to be about 20% amylose by iodine titration. It formed a triacetate and a trimethyl ether, and the methylated starch, on hydrolysis and separation on a cellulose column, yielded chiefly 2,3,6-trimethylglucose with smaller quantities of di- and tetramethylglucoses. The yield of the tetramethyl glucose indicated that the amylopectin portion of the starch had 20 glucose units per non-reducing end group, or was similar to the values found in other starches. Viscosity determinations on the methyl derivative suggested an average molecular weight of about 500,000.

To conclude this review, it seems opportune to summarize the work done on white spruce bark here at McGill University during recent years, in order that the background and previous treatment of the bark used in the present research will be clear. These extractions are outlined in Figure III. Since, in many of the other studies, detailed information could not be obtained because insufficient material was available,

Harwood (22) (23) (24) started this project by extracting 600 pounds of bark with methanol. He fractionated the extract according to Zellner's procedure (Figure I) and studied the wax, wax acids, fatty acids, resin acids and terpenes which were present in these fractions. Later, Murdock (100) investigated the sterol fraction. Andrews (101) has recently completed an investigation of the phlobaphenes (Group III); he isolated a crystalline glucoside, proposed a structure for the aglucone, and showed the presence of another glycoside. Bishop (24) (25) extracted the residual bark with cold and hot water. The extract was combined with the water-soluble material from the methanol extract, and the two were investigated together. These extracts contained free glucose and mannose, complex phenolic glucosides and mannosides, and a large amount of tannin (Figure II). Harpham (102) removed the tannins by adsorption on hide powder, and the sugars by fermentation, and then studied the remaining glycosides by acetylation and chromatography on alumina. He was unable to isolate any crystalline material, but gained considerable insight into the nature of the compounds present. Jablonski (35) treated the water-extracted bark with liquid ammonia, which removed the suberin as acid amides, together with phenolic and carbohydrate material. Sanderson (54) re-extracted the residual bark from the liquid ammonia extraction with cold and hot water, and obtained an extract containing carbohydrates, pectins and tannins. The present report is concerned with the residue left by Sanderson.

FIGURE III

Previous Extractions of the White Spruce Bark (22) (25) (35) (54)



DISCUSSION OF RESULTS

As previously outlined, the bark used in this extraction had already been extracted by methanol, water, liquid ammonia, and again with water. Prior to the liquid ammonia treatment, the bark had been passed through a Mead Mill, so that the particles were all less than 1/8" in diameter. Since the aqueous extractions carried out by Sanderson (54) had all been batch operations, they were not exhaustive, and to complete the removal of the water-solubles, the present research commenced by re-extracting the bark exhaustively with water in a Soxhlet apparatus. This extraction removed a further 1.06% of material, based on the dry weight of the original bark. The aqueous extract was dark red-brown in colour, and was readily attacked by molds if left open to the atmosphere, but the solid extract was not isolated or investigated. The residual bark from this exhaustive extraction is hereafter referred to as "water-extracted bark".

Small-Scale Extractions with Alkali

For further study of the bark constituents, it appeared that extraction with aqueous alkali was required. Since the purpose was to isolate these materials in a state as similar as possible to the one in which they existed in the original bark, a considerable number of small-scale extractions were carried out so that the least destructive conditions could be

selected. The method outlined in the Experimental Section recovered the extracted material from two sources - the alkaline extract itself, and the subsequent aqueous washings of the extracted bark. The second source usually gave the larger amount. In most of the small-scale experiments, the two extracts were worked up together as a matter of convenience, but in the large-scale extractions, they were kept separate. A preliminary experiment showed that sodium hydroxide extracted 18% of the bark, while potassium hydroxide, under the same conditions, removed only 11%. The former was therefore more efficient.

Two methods of precipitating the extracted material were investigated. The first was to acidify the liquor with sulphuric acid; the precipitate which formed only amounted to half of the weight lost by the bark. The soluble portion was obtained by removing the sodium sulphate by dialysis, concentration and then precipitation by the addition of two volumes of alcohol. The second method of isolation involved neutralization with acetic acid, concentration in vacuum, and then acidification with more acetic acid to cause the first precipitate to form. Two volumes of alcohol were then added to the mother liquor and the product collected. The acidification with sulphuric acid gave products of lower ash (7-8% compared to 12-15% by the acetic acid method) but the precipitates were darker in colour. A possible explanation for the discolouration was that the extract could not be

washed completely acid-free, because when the pH of the washings rose above about 4.5, the extract became dispersed in the washings. Acetic acid, on the other hand, was readily removed by washing with aqueous alcohol. A further advantage of using acetic acid for the neutralization was the solubility of sodium acetate in aqueous alcohol. Since sodium sulphate was insoluble in 2:1 alcohol-water mixtures, it had to be removed by dialysis before the alcohol precipitation could take place. While this step could be carried out on a small scale without difficulty, a large-scale dialysis would involve considerable experimental complication. The total weight of the precipitates from the two methods was the same, usually about 5-10% less than the weight lost by the bark; in the acetic acid method, however, the ratio of the two precipitates varied widely with the experimental conditions. Apparently, colloidal phenomena played a greater part in this case. Because the product appeared better, and because the method was more adaptable to large-scale work, acidification with acetic acid was finally adopted.

The first factor to be studied was the duration of extraction. When the extractions were carried out with 2% sodium hydroxide solution on bark samples, the loss in 18 hours was 9.3% of the original weight, while that in 72 hours was 24.7%. These figures seemed to indicate that, while some decrease in the rate of extraction occurred over the longer period, the extracting power of the alkali was definitely not

satiated during the shorter extraction. Since only these two extraction periods were studied, the selection of 72 hours was somewhat arbitrary, but at least this order of time seemed to be advantageous.

The effect of the concentration of the caustic solution on the course of the extraction was then investigated by means of single extractions carried out under otherwise identical conditions. The results are summarized in Table I. As might have been expected, the amount of material extracted increased with increasing sodium hydroxide concentration. However, in selecting the optimum concentration for large-scale extraction, other factors must also be considered. Using the solubility values for sodium acetate in aqueous ethanol given by Seidell (103), it was estimated that 5% sodium hydroxide was the strongest that could be used without precipitating sodium acetate during the addition of the alcohol. Further, when more exhaustive extractions were studied, the difference between 2% and 5% caustic soda was almost eliminated, as shown in Table II. The small amount of material lost in the fourth extraction was apparently largely experimental error, since only a small amount could be precipitated from the liquors. It appeared that three treatments were required for an exhaustive extraction, whether 2% or 5% caustic was used, and it seemed preferable to use the milder reagent.

The next factor to be studied was the ratio of liquor to

TABLE I

Effect of the Sodium Hydroxide Concentration on the Amount of Bark Extracted (a).

Concentration of NaOH soln.	% Weight loss of bark
1%	14
2%	18.5
5%	21
10%	28

(a) Conditions: 10 gm. of bark stirred with 200 ml. of alkali at room temperature for 72 hours.

TABLE II

Effect of Consecutive Extractions on the Weight Loss of Bark (a).

	Cumulative Per cent Extracted	
	5% NaOH	2% NaOH
Extraction 1	21	18.5
Extraction 2	39	36
Extraction 3	44	43
Extraction 4	46	45

(a) Conditions: As in Table I

bark and the minimum volume of washings required to complete the extraction. A constant volume of water was used for each washing, and the number required was recorded. These results are summarized in Table III. A 10:1 ratio was unsatisfactory since the resulting mixture was not sufficiently

fluid for efficient mixing. Further, although increasing the ratio to 30:1 did increase the amount of material extracted somewhat, the increase was not sufficient to permit complete extraction in two treatments. It did not reduce the amount of water washing to a large extent either, so there seemed to be little gain in increasing the volume of liquor beyond 20:1.

TABLE III

Effect of Liquor to Bark Ratio on the Amount Extracted(a)

Ratio	10:1	20:1	30:1
Per cent of bark extracted	13%	18%	23%
No. of washings required	8	7	6

(a) Conditions: 10 gm. of bark stirred with 2% sodium hydroxide for 72 hours.

Since the large-scale extraction had to be carried out without efficient stirring, a small extraction was run in this manner to see how it would affect the results. While the stirred extraction resulted in a weight loss of 18%, this value dropped to 13.5% when the bark was merely allowed to stand in contact with the alkali with no agitation. Hence the decrease in the rate of extraction was quite significant, and suggested the importance of diffusion in the extraction process.

Large-Scale Extraction

Two large-scale extractions were carried out, each using one kilogram of bark and 15 litres of 2% sodium hydroxide solution. The reaction vessel was a large stainless steel pot, which was sealed at the top by a glass desiccator lid. Originally, the pot was to have a stainless steel stirrer built for agitation, but after a conference with Mr. de Montmorency and Mr. Webb of the workshop, it was decided that the time and cost involved were too great to justify this project. Thus the total amount of material extracted from the bark was less than the amount expected from the results of the small-scale extractions. When the extraction vessel was opened, a strong smell of ammonia was evident. Since any ammonium acetate formed by Sanderson would have been eliminated during the exhaustive aqueous extractions, it seemed likely that this odour was caused by the displacement of ammonia from acidic groups in the bark by sodium hydroxide.

The concentration of the neutralized extract was carried out in a large, steam-heated stainless steel still under vacuum, the rate of distillation being adjusted so that the temperature of the liquid in the still pot was kept below 45°C. This method allowed the water to be removed rapidly, but suffered from the disadvantage that the material tended to coat on the walls of the still as the level inside receded. Hence this material was dried from water during the concent-

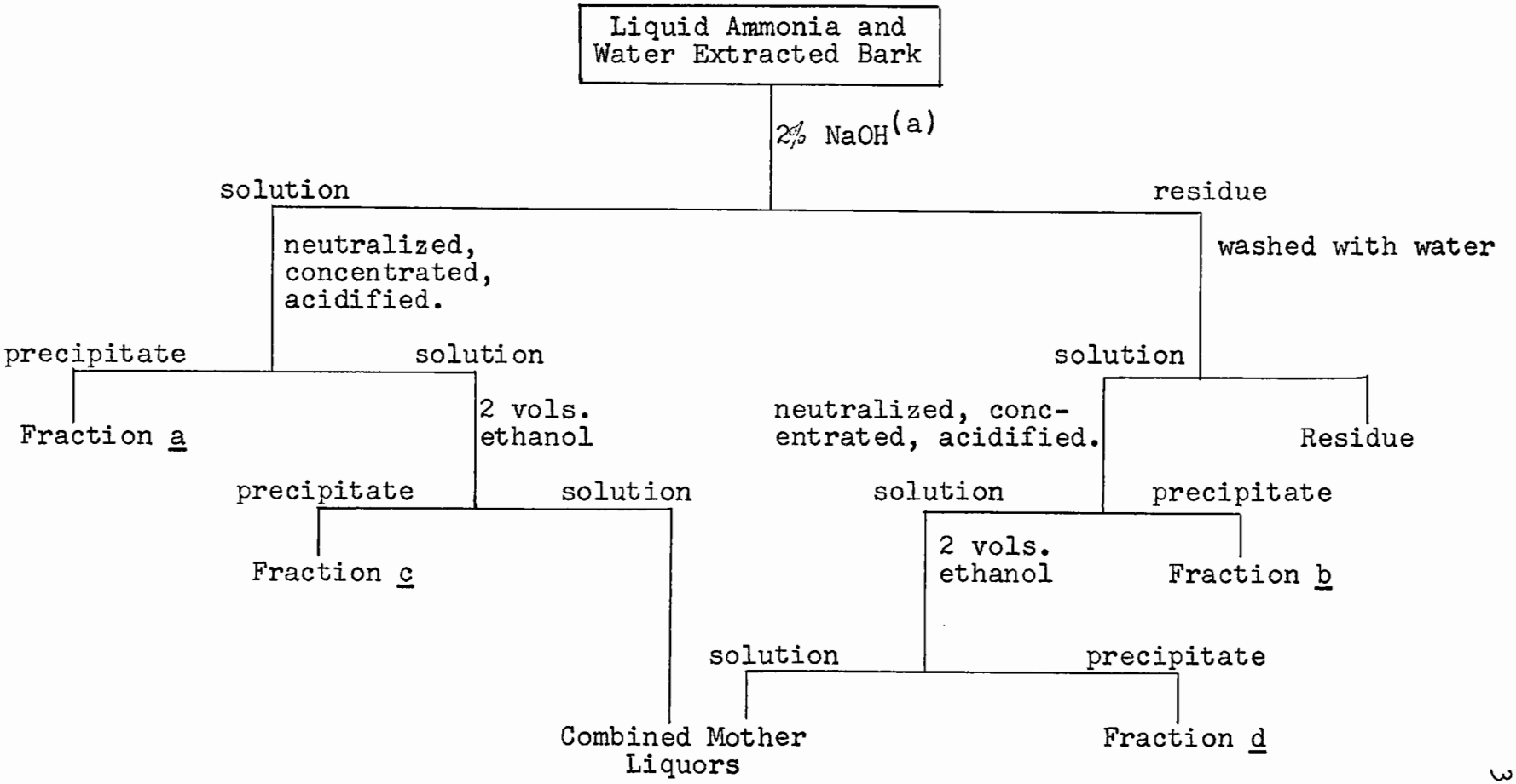
ration, and its colloidal properties during the subsequent precipitation might have been affected.

After precipitation of the extracted material by a method similar to that used for the small-scale extractions, Fractions a, b, c and d were obtained as shown in Figure IV. Since several extractions were carried out on each portion of the bark, these fractions were obtained in several sub-fractions which are listed in Table IV. When the total weight of these fractions was compared with the weight loss in the bark, about 5% of the original material was lacking. To account for this deficit, the combined mother liquors from the extractions and washings were first evaporated under reduced pressure in the large still until all of the alcohol and part of the water was removed. After the residual solution had stood for a few days, a precipitate, which was collected and designated as Fraction E, settled. Fraction F was isolated from an aliquot of the remaining solution by precipitation with acetone after neutralization and removal of inorganic material by dialysis; Fraction G by evaporation of the aqueous acetone mother liquor to dryness. This procedure is outlined in Figure V, and the yields and analyses of these three fractions are given in Table V.

These fractions accounted for only a part of the missing material. The recovery during the complete large-scale extraction was outlined in Table VI, which showed 98.2% recovery if no correction was made for ash, or 94.2% if an ash corr-

FIGURE IV

Alkaline Extraction of the Bark



(FIGURE V)

(a) Total of seven extractions were carried out on two portions of the bark.

TABLE IV

Weights of the Precipitates from the Large-Scale Extractions (a)

1st Extraction(b)

	Fraction Weight in Grams			
	a	b	c	d
Extraction 1	25.80	63.11	19.09	48.37
Extraction 2	8.47	42.10	5.06	2.45
Extraction 3	7.78	35.25	4.54	3.14
Total weight	42.05	140.46	28.69	53.96
Per cent of original bark	2.09%	7.00%	1.43%	2.68%
Total per cent				13.2%

2nd Extraction(c)

Extraction 4	28.80	25.95	28.46	9.40
Extraction 5	19.72	22.37	17.76	29.55
Extraction 6	16.82	6.1	20.95	25.1
Extraction 7	19.17	-	11.55	8.3
Total weight	84.51	54.42	78.72	72.35
Per cent of original bark	4.01%	2.57%	3.73%	3.42%
Total per cent				13.7%

(a) Weights uncorrected for ash

(b) From 946 gm. of water-extracted bark, or 2008 gm. of original bark

(c) From 995 gm. of water-extracted bark, or 2112 gm. of the original bark.

FIGURE V

Isolation of Fractions E, F and G

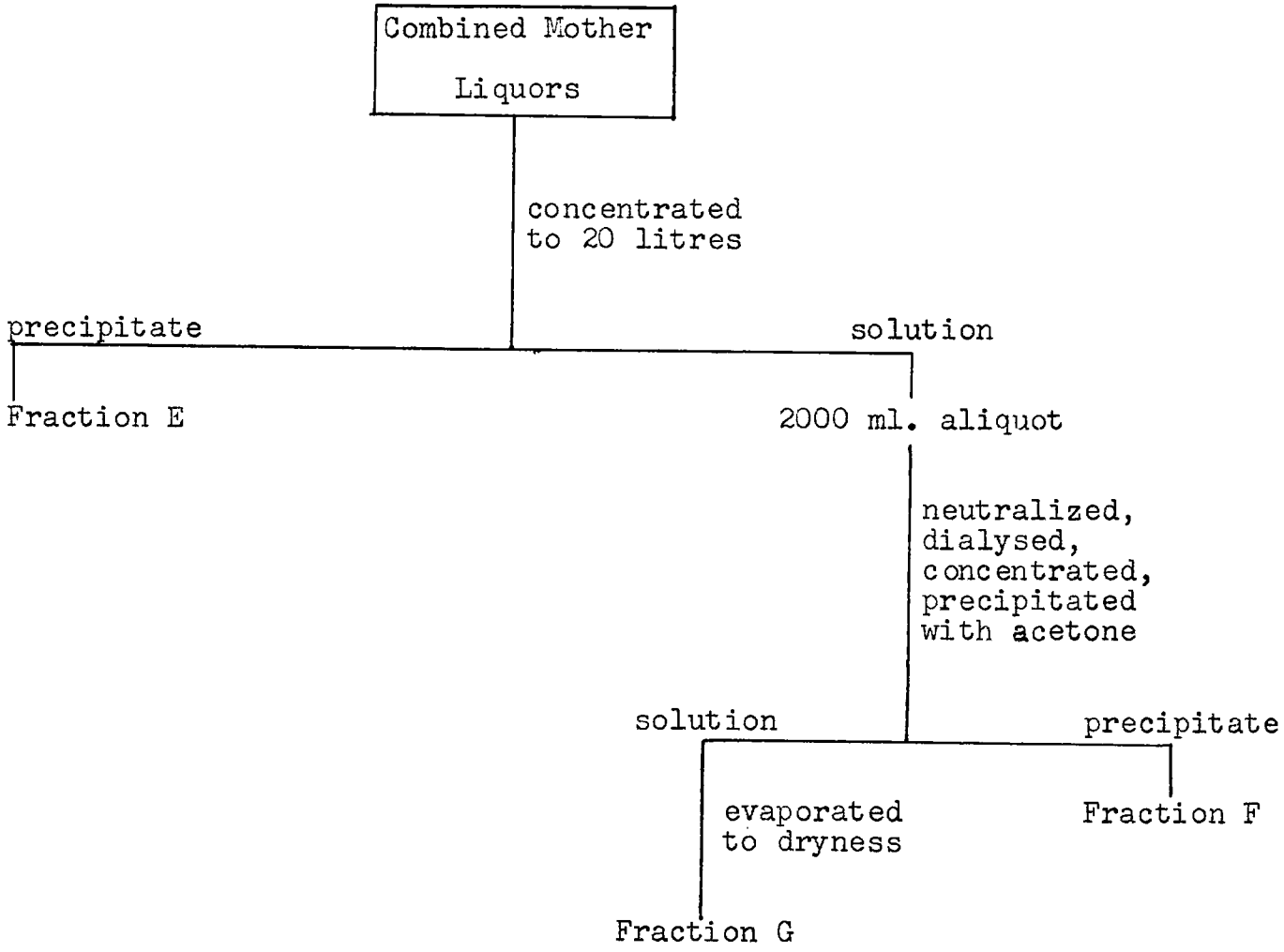


TABLE V

Yields and Analyses of the Minor Fractions

	Fraction E	Fraction F	Fraction G
Yield (gm.)[a]	13.5	21.2	30.2
Yield (%)	0.69	1.09	1.55
Ash (%)	16.6	10.4	18.8
Methoxyl (%)[b]	2.57	2.04	2.19
Furfural (%)[c]	2.33	6.01	8.62
Uronic anhydride (%)[b]	8.98	12.3	4.31

(a) Uncorrected for ash. Extracted from 1949 gm. of moisture-free bark, or 4138 gm. of the original bark.

(b) Corrected for ash

(c) Corrected for ash and for the furfural produced from the uronic anhydride.

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ection was made. The correct value probably lay between these two. The high ash contents in the alkaline extracts resulted from the displacement of hydrogen in acid groups by sodium, and when this salt was ignited under the usual conditions, the resultant product was sodium carbonate, which contained only 43% of sodium. Hence, the ash determinations provided a good basis for comparing weights of different ash contents, but the absolute values obtained might be expected to be a little high. The recovery of 94.2% was considered to be satisfactory, since analytical

TABLE VI

Material Balance for Large-Scale Extraction

	Wt. of Fraction (gm)	Ash %	Ash-free weight (gm)	% of starting material	% of original bark
Bark residue	1294	4.95	1230	66.4	31.3
Fraction A(a)	321.4	18.0	263.6	16.5	7.8
Fraction C(a)	122.4	13.9	104.5	6.28	2.93
Fraction D(a)	111.3	15.6	93.9	5.71	2.69
Fraction E	13.5	16.6	11.3	0.69	0.32
Fraction F	21.2	10.4	18.9	1.09	0.51
Fraction G	30.2	18.8	24.5	1.55	0.73
Total recovery	1914	--	1747	98.3	46.2
Water-extracted bark	1949	4.72	1857	--	47.1

(a) Final composite fractions described later.

errors and difficulty in handling the large quantities involved in the extractions made a quantitative recovery unlikely. The largest source of error probably resulted from the loss of bark particles during the many filtrations and other operations that were carried out in the course of the extractions. For this reason, the amount of material extracted, 14.9% of the original bark, was based on the recovered extract rather than on the weight loss of the bark.

The ash content of each of the sub-fractions was deter-

mined, and the sugars produced on hydrolysis were identified by paper partition chromatography. The ash contents of all the a and b sub-fractions ranged from 17 to 19%, while all those of the c and some of the d sub-fractions fell between 12 and 15%. In Fractions c and d, glucose and xylose were the sugars yielding the strongest spots, but galactose and arabinose were also present, and a streak near the starting line indicated the presence of uronic acid. The principal sugar present in Fractions a and b apparently was glucose, but traces of galactose, arabinose, and xylose were also noted in some of the extracts; a considerable amount of uronic acid appeared to be present. These fractions gave less satisfactory chromatograms than Fractions c and d owing to a tendency to streak, especially around the starting line, probably because Fractions a and b contained more salts and other non-carbohydrate constituents. All the sub-fractions of a and b were combined to give one large fraction, hereafter called Fraction A. The analyses also indicated that the sub-fractions of c and d were quite similar in constitution. However, Fractions d-1, d-5, d-6 and d-7 were much darker in colour than the others, and the last three had higher ash contents. Until the source of these differences could be determined, the above-mentioned d sub-fractions were combined and called Fraction D, while all of Fraction c and the remaining d sub-fractions were united as Fraction C. The weights of the composite fractions A, C and D and

their relationship to the original and the water-extracted bark can be found in Table VI.

The existence of two apparently different water-soluble portions of the extract appears to be caused by incomplete separation of the water-insoluble fraction. It can be seen from Table IV that, in the cases where a dark-coloured sub-fraction d resulted, a greater weight of this portion was obtained relative to the corresponding sub-fraction b. It seemed likely that part of the material normally precipitated on concentration of the extract remained in a colloidal dispersion in these cases; this explanation would be especially plausible for the last three washings of the second extraction, where, owing to the modified procedure, the water washings were not directly preceded by an alkaline extraction, and the resultant low concentration of sodium acetate would produce a medium more favourable to colloidal solutions. In this regard, it should be noted that the terms "water-insoluble" and "water-soluble" have been applied in an arbitrary sense in the foregoing paragraphs. The colloidal properties, which were apparently dominant in this separation, were more drastically affected by the conditions in the surrounding medium than true solution properties would have been. This behaviour explained the fact that even the ratios of corresponding a and c sub-fractions varied somewhat in amount, probably because of variations in the amount of water evaporated from the neutralized extract. Qualitative experiments

showed that the water-insoluble Fraction A was partially dispersed if shaken with distilled water, and this dispersed material was not separated even after prolonged centrifugation at 3500 r.p.m. On the other hand, only an insignificant amount of material became dispersed if this fraction was shaken with 5% sodium acetate.

Examination of Fraction A

Fraction A was a fluffy, dark brown, amorphous solid, insoluble in alcohol, ether, benzene and petroleum ether. It was partially dispersed by shaking with water, and, if wet with water or alcohol, was dispersed by acetone and dioxane. This fraction was soluble in dilute sodium hydroxide, giving black, viscous solutions.

The ash content, 18.0%, was somewhat higher than expected, but the methoxyl content, 1.56%, agreed well with the value of 1.45% obtained by Gleason (43) for an alkaline extract of the same bark prior to the liquid ammonia extraction and was characteristic of the values found by other workers. Fraction A also contained 25.9% of uronic anhydride, and yielded 6.62% of furfural when heated under reflux with hydrochloric acid. However, Norris and Resch (104) showed that pectic acid yielded about 21.5% of furfural by weight under these conditions; based on this factor, the yield for furfural from the uronic anhydride would be 5.57% leaving 1.05% formed by the pentosans present. This result confirmed

that of the chromatographic analysis of this extract, which showed only trace amounts of xylose and arabinose. Thus, the only carbohydrates present in this extract appeared to be based on glucose and uronic acid residues. These analyses are listed in Table VII.

The presence of carbohydrate material in this alkaline extract appears to have been overlooked by the earlier workers, who apparently regarded the extract as bark "lignin" without attempting to remove other possible constituents. Lehmann and Wilke (39) found that their alkaline extracts of pine bark showed an increase in carbon content after treatment with hydrochloric-sulphuric acid; this increase may be explained by hydrolysis and dissolution of carbohydrates, which have a relatively low carbon content (44 to 45%), leaving a residue containing more carbon. Lewis and his associates (9) and Kiefer and Kurth (42) have shown that the material isolated from redwood and Douglas fir bark, respectively, by alkaline extraction, contained a large amount of carboxylic hydroxyl groups. These workers did not eliminate the possibility that the acidic groups were present as uronic acid, and in consequence, there was no justification for assuming carboxyl groups as a part of the bark "lignin" molecule. Since the carbohydrate constituents in this fraction had been given so little attention in the past, the present investigation was directed primarily towards elucidating their nature.

The possibility of reducing the ash content of the fract-

ion was first investigated. After washing a sample of one of the a sub-fractions for a few minutes with cold, dilute hydrochloric acid, 65.5% of the sample weight was recovered as insoluble precipitate with its ash content reduced from 15.0% to 1.84%, while a further 16.1% was recovered by adding alcohol to the acid washings; however, the latter precipitate had a higher ash content (22.6%) than the original. Since the recovery of extract low in ash was not very satisfactory by this procedure, and since subsequent steps would conceivably involve re-solution in alkali, no attempt was made to de-ash Fraction A on a larger scale. Another experiment showed that no ash was removed by dialysis against running water.

The fractional precipitation of this fraction from solution in alkali was now investigated. The extract was stirred with aqueous sodium hydroxide as described for the large-scale extraction, although the ratio of liquor to extract was much less. It was found that 16.5% of the original extract did not dissolve in the alkaline solution, and the significance of this observation became apparent in the later work. Neutralization and concentration of the alkaline solution precipitated only a part of the dissolved material, but the remainder separated on the addition of ethanol. Both the alkali-soluble and alkali-insoluble portions produced glucose and uronic acid on acid hydrolsis, as shown by their paper chromatograms, and little separation of the components appeared

to have occurred. An attempt was made to achieve a fractionation of the alkali-soluble portion of this fraction by neutralizing, concentrating, and acidifying with acetic acid. A further 37% of the weight was precipitated; the addition of small volumes of ethanol to the mother liquor brought about little further precipitation, but when the ethanol concentration was brought up to 33%, a further 26% was collected. Increase of the ethanol concentration to 50 and 80% yielded precipitates of 4 and 3% respectively, bringing the total recovery to 86%. The alcohol precipitation appeared to be occurring over too small a range in concentration to be of any value in fractionation. Again, all the sub-fractions contained both glucose and uronic acid residues, and so this attempt at fractionation was abandoned.

As it seemed quite likely that the uronic acid portion of the extract was pectic in nature, as suggested by Sanderson (54) for his aqueous extract of the bark, Fraction A was extracted with ammonium hydroxide and ammonium oxalate, both good pectin solvents. The former reagent left a residue of 18%, containing 5.52% of uronic anhydride, while the latter left undissolved a portion amounting to 23.5% and having 2.18% of uronic anhydride groups. The extracted material, after precipitation by ethanol, amounted to 84% and 77%, analyzing for 28.8% and 24.3% uronic acid residues, respectively. In both cases, yields of over 100% were obtained, probably because the extracts took up ammonia during the

reaction; since ammonium salts escaped determination in the ash, the correction for ash was probably too small. Both these extracts were quite dark in colour and, after acid hydrolysis, both yielded strong spots of glucose. Thus, although these pectic reagents extracted almost all of the uronic acid portion from Fraction A, the other constituents were dissolved simultaneously, and this method was ruled out as a means of separation. In fact, little difference was observed between the extractions with ammonium hydroxide and ammonium oxalate, and the earlier one with sodium hydroxide.

Since Fraction A was known to contain a large amount of phenolic material, and since glucose was produced on acid hydrolysis, the possibility of the presence of phenolic glucosides was considered. The naturally-occurring glucosides have almost exclusively the beta configuration for the glucosidic carbon atom (105), and can be readily hydrolyzed by the enzyme emulsin. After dissolution in sodium hydroxide, 78% of Fraction A remained dissolved when the solution was neutralized to pH 5, if very dilute solutions were used. Treatment of this solution with emulsin produced no detectable increase in the copper reducing power of the solution after 16 hours. Since the β-glucoside salicin produced 69% of the theoretical increase in reduction within 6 hours when it dissolved in this solution, the activity of the emulsin was not being destroyed by the medium. Hence, the absence of beta glucosides was established, and it seemed that the glucose

residues existed as a polysaccharide.

The acetylation of Fraction A was investigated, using the method of Carson and Maclay (106,107), since this extract was swollen in formamide, but not in pyridine. A fraction amounting to 32% of the starting material and containing 23.0% of acetyl groups proved to be insoluble in the reaction mixture, while a further 19% containing 27.6% of acetyl was isolated after pouring the remaining solution onto ice water. The remaining material appeared to be dispersed in the aqueous solution, and was precipitated neither by the addition of ethanol nor by acidification. The two fractions isolated were not soluble in chloroform or acetone, and were even darker in colour than the original extract. This observation, coupled with the low recovery in the acetylation, indicated little separation would be accomplished by the fractionation of the acetates.

The attempts to resolve Fraction A thus far had involved methods in which all of the constituents of the original mixture would be isolated with little or no change in their chemical structure, but all had failed even to separate the phenolic from the carbohydrate constituents, despite the vast differences in their chemical structures. The destruction of the phenolic portion of the bark while leaving the polysaccharides intact was now investigated. This object was most readily accomplished for woods by bleaching agents, such as chlorine or sodium chlorite. While the application of these

methods to bark has not been investigated in detail, Kiefer and Kurth (42) preferred the sodium chlorite treatment for the fibre fraction of Douglas fir bark, while Jablonski (35) and Sanderson (54) used this reagent in their study of the liquid ammonia and subsequent aqueous extracts of white spruce bark. Sodium chlorite, buffered in acetic acid, was first used by Jayme (108) for the preparation of holocellulose, and the procedure was later modified by Wise (50). Timell and Jahn (109) suggested that Wise's procedure resulted in degradation of holocellulose, while Harwood (110) claimed that severe degradation of the pentosans occurred during the preparation of holocellulose from wheat straw at 75°. On the other hand, Campbell and McDonald (111) showed that little loss of pentosan took place during the chlorite treatment of beech and spruce woods at 50° in a buffer at pH 6.3. These workers did find, however, that sodium chlorite produced an acid-soluble oxidation product of lignin which remained in the holocellulose. A systematic study of the action of sodium chlorite on sugars was carried out by Jeanes and Isbell (112) who found that both the alcoholic hydroxyl groups and glycosidic linkages were inert to attack, but that the reducing group was oxidized, especially in acidic solution. No detailed study of the effect of chlorites on pectins has been reported, although Pallmann and Deuel (113) found that these substances were not degraded by chlorine dioxide.

The bleaching of Fraction A was first carried out on a

small scale at room temperature with the pH maintained at 4.8. The insoluble product, constituting 40% of the original material, was a light cream powder containing 44.0% of uronic anhydride, and the acid hydrolysate revealed that both the glucose and uronic acid had survived the bleaching. The occurrence of glucose residue in the water-insoluble, bleached product confirmed the earlier conclusion that this sugar existed as a polysaccharide structure rather than as a phenolic glucoside. A second fraction of 12% was precipitated by alcohol from the mother liquors; this material was a light brown powder, and its hydrolysate, besides giving the strong spots for glucose and uronic acid, gave a weak indication that xylose was also present. The efficiency of the bleaching treatment was tested by treating a sample of the water-insoluble residue with a second portion of sodium chlorite under the same conditions, and a yield of 83% of water-insoluble and 7% of water-soluble product was obtained; since the ash content was decreased from 22.6% to 16.8% in the reaction, the yield was almost quantitative, and the second treatment was unnecessary. The twice-bleached product contained 42.1% of uronic anhydride.

As the success of these exploratory experiments warranted chlorite bleaching of the bark on a larger scale, 120 gm. of Fraction A was treated in three operations. Instead of suspending the dry extract directly in water, it was first dissolved in sodium hydroxide and then the pH was adjusted with

acetic acid, so that the material was probably in a finer, more reactive condition. Also, better control was kept of the reaction temperature since in the small-scale experiment, after an induction period of several hours, a rapid reaction took place, in which the temperature rose significantly. The yields in this experiment were 46.8% of the water-insoluble material, Fraction A-1, and 10.8% of the water-soluble portion, Fraction A-2; the total being about 5% greater than that obtained from the small-scale experiment.

Almost 50% of the ash-free weight of Fraction A-1 was uronic anhydride (Table VII) and the amount of pectin was presumably large. Milks (114), however, found that the portion of spruce wood periodate lignin remaining water-insoluble after treatment with chlorine dioxide yielded carbon dioxide equivalent to 21.3% of uronic anhydride when heated under reflux with 12% hydrochloric acid, and thus any lignin which remained in the holocellulose would produce high results in the uronic acid determination. Milks found an excess of 10% uronic anhydride in his holocellulose. In the bleaching of Fraction A, the total weight of uronic anhydride in the products was only 2% greater than the weight in the starting material, and it therefore appeared that nearly all the carbon dioxide was yielded by uronic acid residues. The yield of furfural from Fractions A, A-1 and A-2 was almost completely accounted for by that which could be formed from the uronic anhydride groups, and the small differences remaining fell well within the range of variation of the

TABLE VII

Comparison of Bleached and Unbleached Fraction A[a]

	Original	Water-insoluble	Water-soluble
Fraction	A	A-1	A-2
Yield (%)	-	46.8	10.8
Ash (%)	18.0	21.8	19.9
Klason "lignin" (%)	42.7	4.76	9.05
Degree of hydrolysis[b] (%)	30.3	46.5	50.4
Uronic anhydride (%)	25.9	48.6	35.4
Furfural[c] (%)	6.62	11.2	7.47
Furfural (calculated from uronic anhydride) (%)	5.57	10.4	7.61
Methoxyl (%)	1.90	0.49	1.66
Sugars on hydrolysis			
Glucose	strong	strong	strong
Galactose	trace	trace	weak
Arabinose	trace	trace	trace
Xylose	trace	weak	weak
Uronic acid	strong	strong	strong

(a) All analyses corrected for ash
(b) Expressed as per cent of glucose
(c) Uncorrected for the furfural produced by the uronic anhydride.

correction factor used. Since a very weak spot for xylose on the paper chromatograms of the hydrolysates was the only other indication of pentosan in these fractions, pentosan

was only a very minor constituent and was not considered further.

The combined yield of Fractions A-1 and A-2, 57.6%, indicated that 42.4% of Fraction A was destroyed during the chlorite treatment. This result agreed very well with the Klason "lignin" content of Fraction A, 42.7%, although this agreement was to some extent fortuitous, since there was still 4.76% of Klason "lignin" in Fraction A-1. A study was made of the acid hydrolysis of Fractions A, A-1 and A-2 by determining the increase in copper reducing power. Since the mild conditions used, N sulphuric acid at 95° for 6 hours, would have had little affect on pectic materials, it was assumed that all the increase in copper reducing power resulted from hydrolysis of polysaccharides not based on uronic acid units. The total polysaccharide content was therefore calculated as the sum of the reducing sugars produced on hydrolysis and the uronic anhydride. Thus, Fraction A contained 56.2% of carbohydrate material, in good agreement with the yield of 57.6% obtained after the chlorite treatment. For Fraction A-1, the polysaccharide content was 95.1%, while the Klason lignin content was 4.76%, which again indicated that these two constituents accounted for all of the material. For Fraction A-2, the polysaccharides totalled 85.8% of the extract but only 9.05% of Klason lignin was detected, totalling 94.8%. However, in this fraction, which was water-soluble, acid-soluble "lignin" was a strong

possibility since the filtrate from the "lignin" determination was very dark in colour. It should be pointed out that two assumptions were involved in these calculations; that no acid-soluble "lignin" was present in the bleached products, and that the uronic anhydride made no contribution to the copper reducing power of the acid hydrolysate. Since these factors act in opposite directions, the possibility of compensation of errors arose, but it seemed unlikely that good correlations would have been observed if these compensations had been large. In this manner, it was shown that sodium chlorite bleaching was a good method of isolating the polysaccharide constituents of the bark extracts.

Evidence that the pectin was hydrolyzed only very slowly under the conditions used was strengthened by the observation that a rapid hydrolysis of Fraction A occurred during the first four hours, but was followed by a very slow further hydrolysis whose rate showed no sign of decreasing after twelve hours. The first rate, which corresponded approximately to the 30% of reducing sugars obtained from this fraction, probably represented the hydrolysis to the sugars, while the slower rate represented the hydrolysis to uronic acids. The uronic acid constituent in Fraction A-1 was identified as galacturonic acid by simultaneous oxidation and hydrolysis to mucic acid, using the method of Heidelberger and Goebel (115). This method employed a mixture of bromine and hydrobromic acid, and had the advantage that neither

glucose nor galactose were oxidized to the corresponding dicarboxylic acids. The mucic acid, after recrystallization from large volumes of hot water, was identified by mixed melting point. The absence of glucuronic acid was indicated when no potassium acid saccharate was precipitated from the mother liquor by following the procedure of Wolfram and Rice (116). This identification of mucic acid confirmed the earlier hypothesis that the uronic acid was present in this fraction in the form of a polygalacturonic (pectic) acid.

The acetylation of Fraction A-1 was studied, first by the method of Hudson and Johnson (117), using fused sodium acetate and acetic anhydride, and then by the method of Carson and Maclay (106,107), using formamide, pyridine and acetic anhydride. The first method recovered only 46% of the starting material as a product which had become considerably darkened, and had only 28.2% of acetyl groups. When the formamide method was applied, the product was recovered in four fractions, as shown in Figure VI. Two of these were precipitated when the reaction mixture was poured into ice, Acetate I being benzene-insoluble, and Acetate II benzene-soluble. Acetate III was recovered by concentrating the aqueous liquor and adding two volumes of ethanol, while Acetate IV was precipitated after the mother liquor had been concentrated until only the formamide remained as solvent. The yields and acetyl contents of these fractions are shown in Table VIII, the total recovery being 60%. A sample of

FIGURE VI

Separation of the Acetates of Fraction A-1

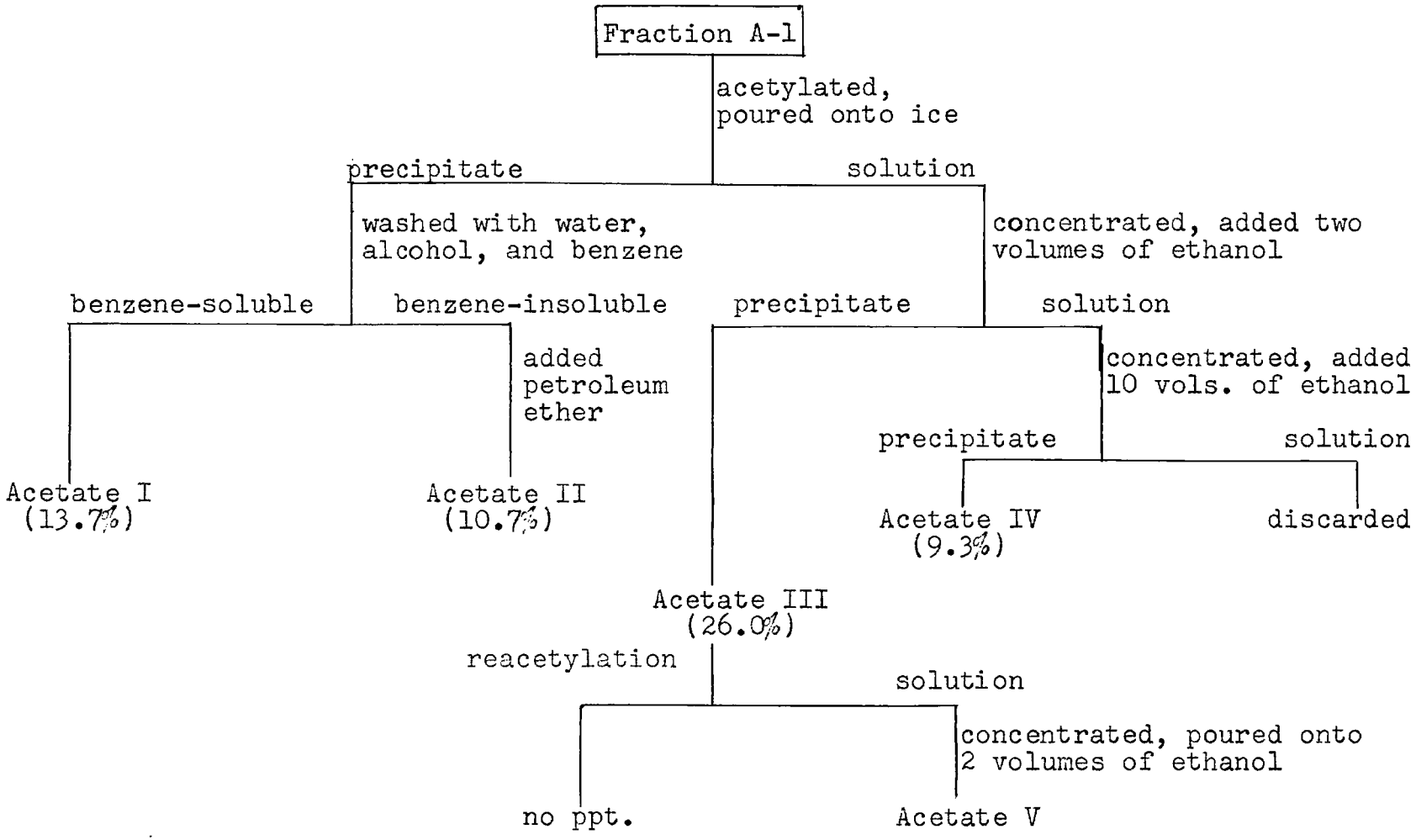

TABLE VIII

Yields and Acetyl Contents of Sub-fractions obtained from the Acetylation of Fraction A-1

Sub-fraction	% Acetyl	% Yield(a)
Acetate I	25.5	13.7
Acetate II	40.7	10.7
Acetate III	23.2	26.0
Acetate IV	28.6	9.3
Total	--	59.7

(a) On an acetyl-free basis; uncorrected for ash.

Acetate III, when reacetylated by the same method, yielded no precipitate when the reaction mixture was poured onto ice water, but 76% of a product containing 32.3% of acetyl groups was precipitated by ethanol from the concentrated mother liquors. The theoretical acetyl content of a pectin diacetate is 32.3%. This reacetylated fraction contained 54.7% of uronic anhydride, equivalent to 80.8% of uronic anhydride diacetate. Thus, the material dispersed in the water appeared to be largely the acetylated pectic acid. Samples of Acetates I, II and III were deacetylated, hydrolyzed and their constituent sugars identified by paper partition chromatography. Acetate I yielded predominantly glucose, but also much smaller quantities of xylose, arabinose and uronic acid, while Acet-

ate II showed only glucose and Acetate III only uronic acid. Thus, it appeared that a partial separation of the pectin and the glucosan was possible by acetylation, but the total recovery was not satisfactory.

The separation of a pectin from other polysaccharides by the dispersion of its acetate in water was first employed by Neubauer (118), and the observation has been confirmed by Milford (119) and Milks (114). These workers used this property as a method for removing pectin as an impurity, but it also appeared suitable for the isolation of the pure pectin as well. The opacity of the liquor indicated that the pectin acetate formed a stable colloidal dispersion rather than a true solution. Carson and Maclay (107) showed that if the acetylated mixture was poured onto ice only partial precipitation of the pectin diacetate occurred, but complete precipitation resulted when 3% hydrochloric acid was substituted for the ice.

The extraction of Fraction A-1 with alkali and reprecipitation from the extract were also investigated. The fact that 28.6% was now insoluble in 2% sodium hydroxide indicated that only a minor portion at most of the alkali-insoluble portion of Fraction A had been destroyed by the sodium chlorite bleach. This residue was high in ash, and the hydrolysate contained small quantities of both glucose and uronic acid. When the ash from this fraction was extracted with a little acetic acid and the soluble portion was tested with ammonium

oxalate solution, calcium oxalate was precipitated and the insoluble portion presumably contained calcium pectate. The glucose in this portion probably arose from bark cellulose which had passed through the filter cloth during the original large-scale extraction. Turning to the alkali-soluble portion, it was all precipitated by neutralization of the extract with acetic acid, followed by the addition of ethanol, yielding another 64.4% of material, and making the total recovery of Fraction A-1 94.0%. This extract also contained both glucose and uronic acid, and, in consequence, the glucose was not only derived from bark cellulose, but also from an alkali-soluble glucosan. Attempts were made to separate this glucosan from the pectic material by fractionation from alkali. When the solution was acidified to pH 1, a precipitate was formed, while two more fractions were precipitated after dialysis and addition of alcohol, as outlined in the Experimental Section. However, each of these fractions on hydrolysis yielded both glucose and uronic acid in about the same relative proportions, and apparently this fractionation did not occur on the basis of chemical structure. Since pectic acid formed a very insoluble calcium salt, the alkaline solution was neutralized to pH 6 with acetic acid, and calcium chloride solution was added, as in the analytical method for the determination of pectin (120). Another precipitate was obtained from the mother liquor after dialysis and the addition of ethanol. Although the chromatograms of the hydrolysates

of these fractions showed that some separation had occurred, this separation was not as good as that obtained by acetylation, and while the former method gave a better recovery of material, the relative weights of the fractions indicated that only low yields of the pure constituents could be achieved if this method were used repeatedly.

The separate acetylation of the alkali-insoluble and alkali-soluble portions of Fraction A-1 was now carried out. The insoluble fraction yielded 55% of water-insoluble material, but this product contained only 5.6% of acetyl groups, while the fraction isolated from the water amounted to 19% and contained only 11.5%; both these fractions contained a considerable amount of ash, and hence this alkali-insoluble material appeared to be of little interest. On the other hand, the alkaline extract yielded only 5% of water-insoluble material, but the acetyl content of 40.9%, indicated the presence of a hexosan. From the aqueous dispersion, an acetate amounting to 48% of the starting material and containing 26.5% of acetyl groups was recovered.

On the basis of these acetylations and alkaline extractions, the most promising approach appeared to be extraction of the alkali-soluble material followed by its acetylation to yield the acetates of the glucosan and the pectic acid. However, when the alkaline extraction was carried out by a procedure slightly modified from the small-scale method, the results differed from those anticipated. Since it was believed

that a large excess of sodium hydroxide solution had been used, the liquor to solid ratio was reduced to one-third for the larger extraction. The other modification involved was the washing of the precipitates with cold, 1% hydrochloric acid in order to reduce the high ash contents. The procedure is outlined in Figure VII while the yields and analyses of the various fractions are given in Table IX. Under these conditions, the alkali-insoluble material, Fraction A-1a, was much greater (40%) than in the smaller experiment (28%) and contained 38.3% of uronic anhydride. After this material had been washed with hydrochloric acid, the aqueous washings were diluted with ethanol to precipitate Fraction A-1b. This light grey powder, amounting to 10.4% of Fraction A-1, contained 94.0% of uronic anhydride after correction for ash, and thus was almost pure calcium pectate, since the ash gave a positive test for calcium. Apparently, the bark yielded not pectic acid, but calcium pectate, during the alkaline extraction, and this substance was removed, not by solution in the alkali, but by the formation of a colloidal dispersion. In consequence, the amount extracted depended on the volume of the solution rather than on the concentration of the alkali. When the extract was washed with acid, some of the calcium was removed, and the pectin was much more soluble in the subsequent washing with water. To remove the residual pectin from Fraction A-1a, the acid-washed material was extracted three times with water, leaving a residue, Fraction A-1c, of 21.5%

FIGURE VII

Fractionation of Fraction A-1 by Alkaline Extraction

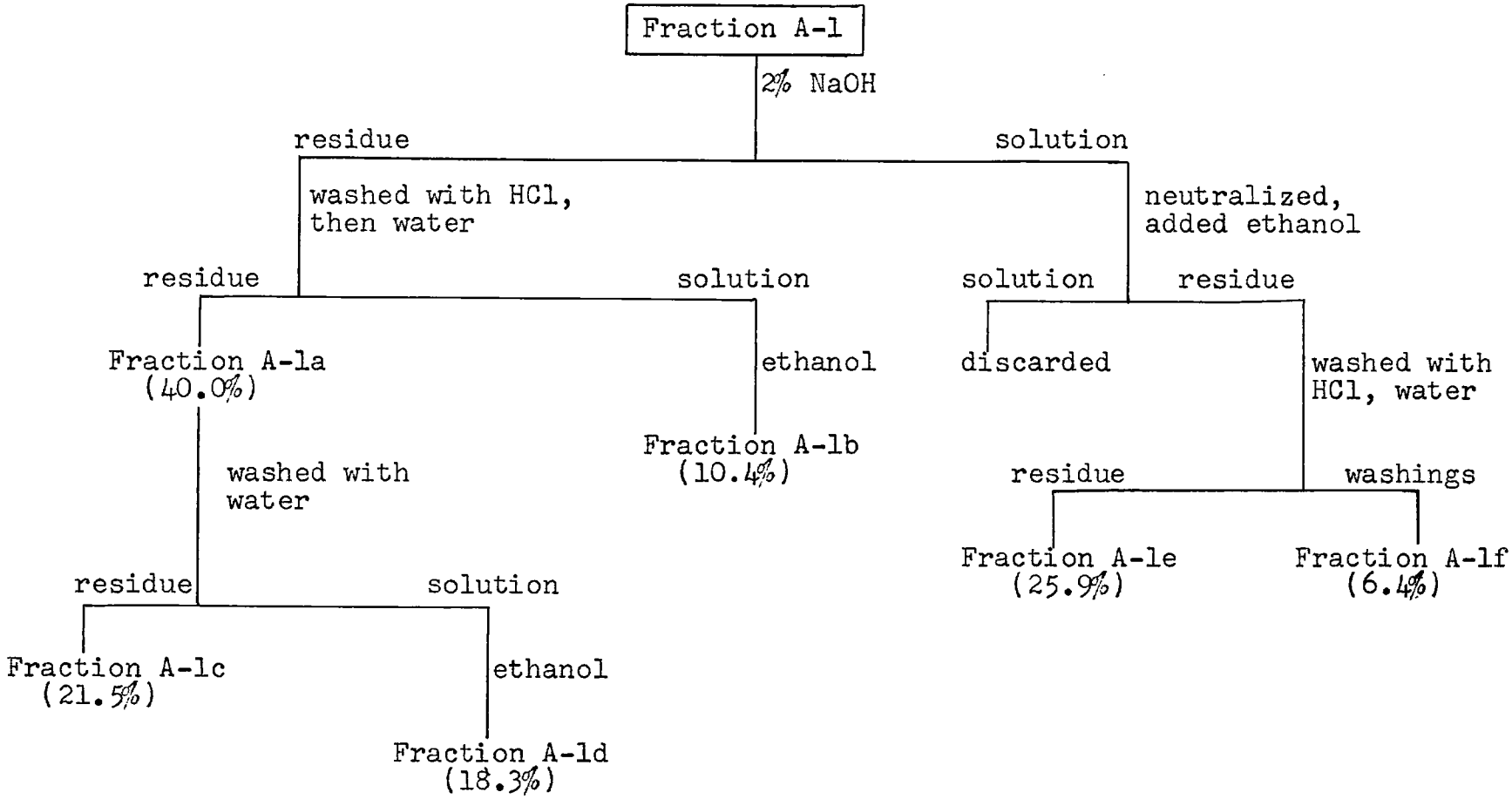

TABLE IX

Yields and Analysis of the Precipitates obtained from the Alkaline Extraction of Fraction A-1

Fraction	Ash (%)	Yield[a] (gm.)	Yield[b] (%)	Uronic anhydride[b] (%)
A-1a	23.8	18.8	40.0	38.3
A-1b	16.0	4.9	10.4	94.0
A-1c	33.1	15.7	21.5	5.28
A-1d	6.55	8.6	18.3	93.8
A-1e	2.14	12.2	25.9	29.7
A-1f	18.9	3.0	6.4	55.5
Total recovery				
	--	40.4	82.5	--

(a) Extracted from 60.0 gm. of Fraction A-1, or 47.0 gm. on an ash-free basis; weights are given on an ash-free basis.

(b) Corrected for ash.

of the original Fraction A-1, containing only 5.28% of uronic anhydride. The aqueous extracts yielded Fraction A-1d which amounted to 18.4% of Fraction A-1, had 93.8% of uronic anhydride, and containing calcium. These two calcium pectate fractions, A-1b and A-1d, appeared to differ only in their ash contents and were combined. Hydrolysis and paper partition chromatography of this combined fraction gave no indication whatsoever of the presence of glucose.

Qualitative tests for calcium were carried out on the ash from the water-extracted bark left by Sanderson, the residual bark from the alkaline extraction, and a bark sample which had been exhaustively extracted with alkali. A strongly positive result was obtained in each case. Similar tests on Fractions A, A-1 and A-1b indicated that these also contained calcium. Gleason (43) had earlier identified calcium oxalate as present to the extent of 8.5% in an alkali extract of the white spruce bark prior to the liquid ammonia extraction. It was possible that the alkali-insoluble Fraction A-1b consisted largely of calcium oxalate although this possibility was not investigated. In any event, the isolation of the calcium pectate from the bark indicated that the insolubility of the "protopectin" resulted from its presence as a calcium salt rather than from chemical bonding to other bark constituents.

The alkaline extract (Figure VII) was obtained in two fractions - Fraction A-1e as the residue and Fraction A-1f from the mother liquor. Fraction A-1e showed only glucose and uronic acid on hydrolysis and thus contained the bulk of the glucosan components of Fraction A-1, together with about 30% of pectin (Table IX). Fraction A-1f, which was only 6.4% of Fraction A-1, was apparently mainly pectic, since it contained 55.5% of uronic anhydride, and gave only very weak spots of glucose after hydrolysis and chromatography. Later, an acetylation of this material indicated

that insufficient glucosan was present to warrant its recovery, since only 13% of the original was precipitated when the reaction mixture was poured onto ice, while a further 43.7% was recovered from the aqueous washings. The acetyl values of these two fractions, 32.2% and 19.5% respectively were somewhat lower than those usually obtained by this method, possibly owing to the presence of non-carbohydrate material.

In the earlier oxidation-hydrolysis of Fraction A-1, the mucic acid recovered accounted for only 20% of the total uronic acid present, and this experiment was therefore repeated on the isolated calcium pectate. In this case, a yield of 65% of the theoretical was obtained, which was more satisfactory, since Wise and Peterson (121) obtained only 75% of mucic acid by the nitric acid oxidation of galactose. Pectic acid was prepared from the calcium pectate by extraction with ammonium oxalate solution, separation of the precipitated calcium oxalate, and reprecipitation of the pectic acid with acidified ethanol. Further reprecipitations, first of the calcium and then of the ammonium salt with ethanolic hydrochloric acid, as suggested by Anderson (84) eventually yielded a white powder, containing 94.7% of uronic anhydride, only 0.52% of ash, and giving solutions in ammonium hydroxide which were clear enough to be observed on the polarimeter. The observed specific rotation of $+265^{\circ}$ compared favourably with the value of $+264^{\circ}$ obtained by O'Dwyer

(81) for a pectic acid isolated from beechwood, although it was a little higher than others obtained by Anderson and his co-workers (83,84,86,87) from other woods.

These pectins, together with those isolated later from the acetates of Fractions A-1e and A-1f accounted for 42.8% of Fraction A-1, neatly checked the uronic acid content of 48.6%, and corresponded to 1.56% of the original bark. Since no other constituent containing uronic anhydride groups was revealed during the investigation of Fraction A, it was quite likely that all of this group was present in the form of pectin, which would then amount to 2.02% of the bark. Other sources of pectin in the bark included that present in Fractions C and D, the 1.9% found by Sanderson in his Fraction A, together with that in his water-soluble acetates from his fractions B and C, and probably part of the 3.16% of uronic anhydride remaining in the alkali-extracted bark. It can be seen that if the pectin from all these sources were positively identified, pectin would likely be recognized as a major constituent of white spruce bark. Even the amount already isolated, is enough to show that this material occurs to a much greater extent in bark than in wood.

The glucosan portion of the extract was obtained in a fairly pure condition as its acetate from the acetylation of Fraction A-1e, using formamide as a swelling agent and pyridine as catalyst. Four fractions were obtained as shown in Figure VI, and these were assigned the corresponding numbers

Ie-IVe, their yields and acetyl contents being listed in Table X. Both the benzene-insoluble Acetate Ie and the benzene-soluble Acetate IIe showed strong spots of glucose after saponification, hydrolysis and paper partition chromatography, but Acetate Ie also showed the presence of some uronic acid, which did not appear in Acetate IIe.

TABLE X

Data on the Acetylation of Fraction A-1e

Fraction	Yield (gm.)[a]	Yield (%)[b]	Acetyl (%)
Acetate Ie	5.0	27.0	40.6
Acetate IIe	6.17	31.4	43.9
Acetate IIIe	2.63	18.4	23.1
Acetate IVe	1.87	13.6	19.8
Total yield	15.67	90.4	--

(a) From 11.0 gm. of Fraction A-1e; uncorrected for ash

(b) Calculated on an acetyl-free basis.

The latter fraction contained only 2.75% of uronic anhydride groups, and although its chloroform solution was somewhat coloured, a specific rotation of +178° was observed. According to the table given by Degering (122), the specific rotations of starch acetates ranged from +155° to +190°, since the source of the starch, its degree of acetylation, and the conditions for measurement of the rotations varied considerably.

The above value for the bark glucosan fell within this range, and suggested that this polysaccharide had the alpha configuration and belonged to the starch group.

In order to isolate more of this starch acetate, Acetate Ie was reacetylated by the same procedure and a portion of the water-precipitated product amounting to 11.5% of Fraction A-le became soluble in benzene, while 12.5% still remained benzene-insoluble. These sub-fractions had acetyl contents of 42.7% and 39.8% respectively. Some further material remained dispersed in the water, but was not recovered. The two benzene-soluble acetates were combined, and, as their acetyl content was slightly below the theoretical value of 44.8% for starch triacetate, a reacetylation was carried out. The product still contained only 43.4% of acetyl groups; this low acetyl value was later explained, however, when an acid hydrolysis of the deacetylated starch showed it to contain 7.9% of unhydrolysable material.

The reason for the existence of these two starch acetate fractions, differing in their solubilities in benzene, is not yet clear, and such partial solubility has apparently not been reported before. The solutions were possibly colloidal in nature, since the dry acetates dissolved much less readily. Both the soluble and insoluble acetates, on deacetylation, gave the characteristic blue colouration of starch with iodine, whereas Fraction A-le, still containing a considerable amount of pectin gave a red-purple colour. Both gave glucose as the

only constituent sugar on hydrolysis and chromatography. Possibly the difference arose from the slightly lower acetyl content of the insoluble material, or from structural differences such as degree of branching or of polymerization. However, both fractions were well characterized as starch acetates.

This isolated starch acetate constituted 15.1% of Fraction A-1, or 0.55% of the original bark, although in this case also, the isolation procedures were not quantitative. Unfortunately, there is no starch analysis, equivalent to the uronic anhydride determination in the pectin field, which could be used to establish the maximum quantity present. It appears that starch was present to about the same extent in this bark as was found by Campbell and his co-workers (94, 98) for certain woods. No previous isolation of starch from bark was revealed in a search of the literature.

The isolated starch and pectin fractions accounted for 57% of Fraction A-1, while a further 21% was left as the alkali-insoluble residue, Fraction A-1c. The remaining 22% was presumably also made up of these two polysaccharides, together with the residual "lignin" and other minor constituents. The maximum value for the starch content of this fraction could be estimated by assuming it to be made up of the four isolated constituents - the alkali-insoluble Fraction A-1c, Klason lignin, pectin, and starch. Since the first three totalled 73.7%, by difference, the starch made up 26.3% so that at

least 60% of the total was recovered by the separations described.

Samples of the pectin and starch acetates were deacetylated to permit of their further characterization. The pectin acetate, partially dissolved in acetone, was treated with aqueous sodium hydroxide for 12 hours. A purified pectic acid was isolated in 78% yield from the product by three reprecipitations as its calcium salt followed by two reprecipitations from ammonium hydroxide with acidified ethanol. The product, slightly less pure than the one isolated previously, contained 84.8% of uronic anhydride and had a specific rotation of $+226^{\circ}$ in 1% ammonium hydroxide solution. This result confirmed the earlier evidence that the acetate which dispersed in water was a pectin acetate, and stressed the usefulness of this method for the isolation of pectins. Starch and pectin have been considered difficult to separate from each other, but the acetylation procedure used on Fraction A-1e resulted in the isolation of pure samples of both in good yield.

The deacetylation of the starch triacetate proved surprisingly difficult. The first treatment, following the method of Wise et al (123), involved the use of dioxane solution of the acetate and alcoholic potassium hydroxide. Even after a subsequent treatment with aqueous sodium hydroxide, 3% of acetyl groups remained in the product, and another saponification with aqueous alkali was required to complete the deacetylation. The product, obtained in 94% yield, contained only

2.90% of uronic anhydride and 1.10% of ash, and its specific rotation was +138° in 1% sodium hydroxide solution, comparing well with the value obtained for starch isolated from oak sapwood by Campbell (94).

The hydrolysis of this starch by acid and by enzyme was studied. On acid hydrolysis, 8% of the starting material did not dissolve, and glucose was the only constituent sugar in the solution. The identity of this glucose was confirmed by conversion, first to the phenylosazone (124) and then to glucose phenylosotriazole, as suggested by Hann and Hudson (125). This crystalline product did not depress the melting point of an authentic sample. The enzymatic hydrolysis was carried out using an alpha amylase enzyme, buffered at pH 5.2, and following a modification of the method of Pigman (126). After this treatment, the solution no longer coloured iodine. The portion of the product soluble in 70% ethanol was studied by paper chromatography, and a weak spot of glucose and a strong one of maltose were identified. Another strong spot, having half the mobility characteristic of maltose, and probably indicating maltotriose was also observed. The appearance of glucose in this hydrolysate indicated the presence of maltase as an impurity in the amylase sample used, since a pure crystalline alpha amylase isolated by Meyer and his associates (127) did not hydrolyse terminal glucose units, and left maltose and maltotriose unaffected.

Investigation of Fractions C to G

A small portion of Fraction C (Figure IV), when bleached with sodium chlorite as described for the bleaching of Fraction A, produced a water-insoluble Fraction C-1 in 8% yield, and a water-soluble Fraction C-2 in 68% yield. As 76% of Fraction C was not destroyed by sodium chlorite, it appeared that this portion was polysaccharide in nature. A series of analyses conducted on Fractions C, C-1 and C-2 are listed in Table XI. The high ash content and the chromatographic analysis of Fraction C-1 indicated that it was quite similar to the insoluble material formed on bleaching Fraction A, although an insufficient amount was obtained in this experiment to permit of a detailed examination. Fraction C-2, on the other hand, was similar to the water-soluble bleached material studied by Sanderson (54). The furfural reported for Fraction C-2 was equivalent to 35% of pentosan if the conversion factor for mixtures of xylose and arabinose was applied. It was also interesting to note that the methoxyl content increased slightly during the bleaching, indicating that this group was bound in residues stable to the treatment, possibly the 4-methoxyglucuronic acid structure found in wood hemicelluloses.

The large number of glucose residues in Fraction C-2 might be present as starch, as was the case in Fraction A, in which the starch was only partially precipitated after the neutralization and concentration of the alkaline extract. To

TABLE XI

Yields and Analyses of Unbleached and Bleached Fraction C[a]

Fraction	C	C-1	C-2
Yield (%)	-	7.7	66
Ash (%)	14.0	24.5	10.0
Methoxyl (%)	1.73	-	1.85
Uronic anhydride (%)	15.6	-	20.3
Furfural[b] (%)	14.8	-	22.1
Sugars on hydrolysis:			
Glucose	strong	strong	strong
Galactose	weak	absent	moderate
Arabinose	moderate	trace	moderate
Xylose	strong	weak	strong
Uronic acid	weak	strong	weak

(a) All analyses corrected for ash.

(b) Corrected for the furfural produced by the uronic acid.

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explore this possibility, a buffered aqueous solution of Fraction C-2 was treated with alpha amylase enzyme as previously described, and the resultant solution imparted no colouration to iodine, whereas the original sample produced a definite purple colour. The higher molecular weight material was precipitated from solution with alcohol, and the mother liquor was studied by paper chromatography. The same three spots were observed as in the starch hydrolysis from

Fraction A, although not as strong in this case. When the material precipitated by alcohol was hydrolysed by acid, the glucose spots from the hydrolysates were only slightly weaker in relation to the other sugars than in the original material. These results indicated that a highly branched starch was present. Whelan and Roberts (128,129) found that when alpha amylase acted on a linear starch (amylose), the sole products were maltose and maltotriose, but if a branched starch (amylopectin) was used, dextrins of five to eight glucose units were also present in the hydrolysate. These workers concluded that this enzyme was incapable of cleaving α-1,6 linkages and also the α-1,4 linkages adjacent to an α-1,6 linkage.

Fraction D was not investigated, while the only further work on Fractions E-G was the analyses listed in Table V. It has been postulated that these fractions, not precipitated by ethanol, might contain araban, but the low yields of furfural indicated that this possibility was unlikely. An attempt to identify any sugars present by the chromatographic method failed because the hydrolysates streaked on the paper; these fractions apparently did not contain a significant amount of carbohydrate material.

Investigation of the Bark Residue

As the large-scale extraction did not completely remove all the material soluble in alkali, a finely-ground portion of the bark residue was exhaustively re-extracted with 10%

sodium hydroxide. This operation removed a further 17.0% of the bark, bringing the total extractable material to 50.6% of the water-extracted bark, or 23.8% of the original. Although somewhat more than was removed by the small-scale extractions, the result checked well with the material isolated from the extracts, 22.4%. The residue was then bleached with sodium chlorite at 50°, and 78.9% of nearly white material was recovered. A series of analyses carried out on the various bark residues is summarized in Table XII. Anderson (83) reported that part of the pectin was not extracted from various woods by ammonium hydroxide until after the wood was chlorinated, and the present bleached bark was accordingly extracted with this reagent. Although the uronic acid content of the sample was reduced from 3.16% to 2.05% by this treatment, the material isolated from the extract did not have the properties of pectin which apparently had all been removed by the alkaline extractions. The presence of glucose as the main sugar in the bleached bark residue was shown by acid hydrolysis and conversion to glucosotriazole by the method outlined earlier; this derivative was identified by the mixed melting point method.

The present research has shown that a considerable portion of the material extracted from white spruce bark by alkali is polysaccharide in nature, and that these polysaccharides can be isolated from the extract by sodium chlorite bleaching. The water-insoluble portion of these polysaccharides has been

TABLE XII

Analyses of the Various Bark Residues[a]

	Water-extracted bark	Residue from large-scale extraction	Residue from exhaustive alkaline extraction	Chlorite-bleached bark
Yield from water-extracted bark (%)	-	66.4	49.4	39.9
Fraction of original bark (%)	47.1	31.3	23.2	18.8
Moisture (%)	12.3	6.58	4.25	2.20
Ash (%)	5.41	5.30	3.93	1.97
Klason "lignin" (%)[b]	-	-	26.7	7.78
Methoxyl (%)	2.19	2.44	2.89	1.48
Uronic anhydride (%)	7.28	3.71	2.81	3.16
Furfural (%)[c]	5.45	3.91	3.81	4.46
Sugars on hydrolysis:				
Galactose	weak	trace	absent	absent
Glucose	strong	strong	strong	strong
Arabinose	weak	trace	trace	trace
Xylose	weak	weak	weak	moderate
Uronic acid	moderate	weak	weak	weak

(a) All analyses corrected for ash and moisture. (b) Single determinations only.
(c) Corrected for the furfural produced by the uronic anhydride.

shown to be predominantly pectin and starch, and pure samples of each have been isolated and characterised. A precursory examination of the water-soluble material indicated that it was a very complex mixture of polysaccharides which would require detailed study in order to isolate pure constituents. The bark remaining after the alkaline extraction was found to be based chiefly on glucose residues.

EXPERIMENTAL RESULTS

ANALYTICAL METHODS

All the determinations were carried out in duplicate, unless otherwise stated, and the results reported are the average values.

MOISTURE CONTENT

Bark samples of from 1 to 2 gm. were heated in an oven at 105°C. for 16 hours and weighed. The loss in weight was taken to be equivalent to the moisture content of the sample.

ASH

The method of Niederl and Niederl (130) was used. Samples of 10 to 20 mg. were weighed into platinum boats and ashed at 600 to 700°C. for 30 minutes. For bark and larger extracts, however, the sampling error was too great to allow the use of these small samples, and the method was modified slightly. Samples of 0.1 to 0.2 gm. were weighed into porcelain crucibles and ignited in a muffle furnace at 650°C. for 12 hours and the cooled crucible reweighed.

SOLIDS IN SOLUTION

Aliquots of 20 ml. of the solution were transferred to tared weighing bottles and evaporated to a syrup at 80°C. in an oven. They were then heated in the oven at 105°C. overnight.

SEALED TUBE HYDROLYSES AND PAPER PARTITION CHROMATOGRAPHY

Samples of 25 to 30 mg. were placed in glass tubes with 1 ml. of N sulphuric acid. The tubes were then sealed and heated at 95°C. in an oven for 16 hours. The solid material remaining undissolved was separated on a centrifuge and the mother liquor was neutralized to Congo Red paper with solid barium carbonate. This precipitate was also removed on the centrifuge. The solution, after being concentrated to a syrup under reduced pressure, was dissolved in 0.1 ml. of water, and spots of about 0.01 ml. were applied to the starting line of a sheet of Whatman No. 1 filter paper (6" x 22") using a platinum wire loop. A spot of a solution of known sugars was placed adjacent to the spot of the hydrolysate, the paper was hung in a Fisher chromatographic tank for an hour to equilibrate, and then was developed with a solution of ethyl acetate-pyridine-water (5:2:5) (131). This development lasted for about 16 hours, and the chromatogram was then air dried. The paper was sprayed with either aniline phthalate (132) or aniline phosphate (133) dissolved in butanol saturated with water and was heated to 100°C. for a few minutes. Under these conditions, these sprays give pink spots with pentoses, and brown spots with hexoses.

FURFURAL

Samples were distilled with 12% hydrochloric acid at the rate of 3 ml. per minute for 100 minutes in the apparatus described by Bray (134) as recommended by TAPPI Standard

Methods (135).

The furfural in the distillate was determined by the TAPPI modification of the bromide-bromate method. In this method, one mole of furfural consumed one mole of bromine. Since different factors were applied in the conversions of furfural to pentosan for arabinose and xylose, the results were reported as per cent furfural, as the paper chromatograms indicated both of these pentose sugars to be present in some of the fractions. The yield of furfural was calculated by substitution in the expression

$$\% \text{ furfural} = \frac{(V_2 - V_1) \times N \times 0.048 \times 100}{W}$$

where V_2 = volume of thiosulphate used in the blank determination

V_1 = volume of thiosulphate used for the sample

N = normality of the sodium thiosulphate solution

W = weight of sample in grams

0.048 was a factor representing the weight of furfural in grams equivalent to 1 ml. of N sodium thiosulphate

The values were corrected for the furfural produced by the uronic acids present on the basis of a furfural yield of 21.5% from glucuronic acid as reported by Norris and Resch (104).

URONIC ANHYDRIDE

At first the method of Browning (136) was used, but this method required inconveniently large samples so that the weight of carbon dioxide evolved was large enough to be weighed acc-

urately. A semimicro method, recently published by Johansson, Lindberg and Theander (137), was used in all later work.

In this method, samples estimated to contain 10 to 25 mg. of uronic anhydride were weighed into the distilling flask, and 25 ml. of 12% hydrochloric acid and a boiling chip were added. The apparatus was flushed with a stream of nitrogen at room temperature for 20 minutes and at 70 to 80°C. for 10 minutes. Then 25 ml. of an aqueous solution, 0.2N in sodium hydroxide and 0.05M in barium chloride, was added to the absorption tube, the bath temperature was raised to 140 to 145°C., and the sample was heated under reflux at this temperature for 4 hours, with a constant flow of nitrogen. At the end of this period, 10 ml. of 2N ammonium chloride was added to the absorption tube. The precipitate was removed on a sintered glass funnel and was washed, together with the tube, with 50% aqueous ethanol. The total precipitate was then dissolved in 5 ml. of 0.5N hydrochloric acid, and the solution was sucked into a 100 ml. flask and the tube and funnel washed with water. Then 10 ml. of M/6 iodic acid was added to the flask. It took several hours for the precipitate of barium iodate to form. The mother liquor was removed by immersion filtration and then the precipitate was washed four times in absolute ethanol, and disintegrated in about 2 ml. of ethanol. Potassium iodide and hydrochloric acid solutions were added, and the liberated iodine was titrated with

0.05N sodium thiosulphate using a starch indicator. The uronic acid anhydride content was calculated from the expression

$$\% \text{ uronic anhydride} = \frac{V \times N \times 194 \times 100}{W \times 12 \times 1.019}$$

where V = the volume of sodium thiosulphate

N = the normality of the sodium thiosulphate

W = the weight of sample in milligrams

The factor 1.019 was a correction for the iodic acid remaining adsorbed on the precipitated barium iodate.

Mr. Watts was able to recover carbon dioxide quantitatively by this method from sodium carbonate, but a commercial sample of galacturonic acid, apparently impure, gave low results. After being purified by recrystallization of its sodium calcium salt, as recommended by Isbell and Frush (138), this sample gave a satisfactory yield of carbon dioxide. A blank determination on glucose yielded carbon dioxide equivalent to 0.98% uronic anhydride. This result compared well with the value of 0.88% observed by Whistler, Martin and Harris (139). Since the method was used directly on acetates for the first time, a blank on pentaacetylglucose was also determined. In this case, carbon dioxide equivalent to 0.51% uronic anhydride was evolved, or, 1.1% on the basis of glucose itself.

ACETYL

Acetyl was determined by Clark's method (140), in which the sample was deacetylated by heating with alcoholic alkali, and the liberated acetic acid was separated by steam distillation of the acidified solution. The amount of distillate was increased from 50 to 100 ml., and the correction factor was ignored, as suggested by Jablonski (35).

METHOXYL

Methoxyl analyses were carried out by the Penniston and Hibbert modification (141) of the Vieboch and Schwappach method (142). The scrubbing solution used was that recommended by Friedrich (143), consisting of a mixture of equal volumes of 5% aqueous sodium thiosulphate and 5% aqueous cadmium sulphate.

KLASON LIGNIN

Klason lignin was determined by the Standard TAPPI Method (144), which used a digestion period of two hours at 18 to 20°C. with 72% sulphuric acid, followed by dilution to 3% acid with water, and a further hydrolysis at the reflux temperature for 4 hours.

COPPER REDUCING POWER

The reducing power of sugar solutions was determined by the Somogyi modification of the Shaffer-Hartman method, as outlined by Browne and Zerban (145). The mixture of the copper reagent and sugar solutions was heated for 40 minutes

in all cases, and the reagent was calibrated using glucose as standard. All values were reported on this basis. The calibration curve was linear between 0.2 and 2.0 mg. of glucose per 5 ml. of solution, and so, wherever possible, the sugar concentration of the solution was adjusted by dilution to within this range before the determination.

CALCULATION OF YIELDS

Sanderson (54) summarized the proportions of the bark extracted by the earlier workers (see Figure III) and showed that material amounting to 47.1% of the original bark had been removed up to the end of the extraction with liquid ammonia. He removed 4.68% in the subsequent large-scale extraction with water, and a further 1.06% was removed in the exhaustive water extraction at the start of this research. Thus the bark used as the starting point for the present alkali extraction represented 47.2% of the original bark used by Harwood for methanol extraction. All yields have been multiplied by a factor 0.472 to convert them to the basis of the original bark.

EXHAUSTIVE EXTRACTION OF THE BARK WITH WATER

The residual bark from Sanderson's aqueous extractions, in portions of 350 to 400 gm., contained in a cotton bag, were continuously extracted with water in a large Soxhlet apparatus for 3 days, at which time the material siphoning over was colourless. The bark was air-dried, while the extracts were combined and stored under toluene. The total

solids in the combined extract was determined, and it was found that from 3780 gm. of bark, 83.6 gm. (or 1.06%) of material had been extracted.

SMALL-SCALE EXTRACTIONS OF THE BARK WITH ALKALI

The bark samples (usually about 10 gm., dry weight) were accurately weighed and placed in 500 ml. round-bottom flasks which were set up for mechanical stirring. A sodium hydroxide solution of the desired concentration and volume was added, and then each flask was flushed with purified nitrogen gas. The mixture was stirred for 72 hours, occasionally washing down the material which splashed onto the side walls of the flask. When the extraction was completed, the reaction mixture was separated on the centrifuge into the mother liquor and the residual bark. The residue was washed by stirring in the centrifuge bottle for a few hours with 50 ml. volumes of water until the washings were only slightly coloured. About 6 washings were usually necessary. The bark was washed twice with 95% ethanol, was dried in vacuum over phosphorus pentoxide overnight, and weighed. Corrections were made for the moisture content before and after extraction. The extract and washings were treated by one of the two procedures below.

A. PRECIPITATION WITH SULPHURIC ACID

The extract was acidified with 10% sulphuric acid until a pH of 2 was reached. Although a colour change occ-

urred between pH 3 and 5, the precipitate only separated cleanly at the lower pH. The precipitate was collected on the centrifuge, was washed once with distilled water, twice with alcohol and twice with benzene, and then was dried overnight in vacuo over phosphorus pentoxide. The acidified mother liquor from the precipitation was dialysed in a cellophane membrane against running water until a negative test for sulphate ion was obtained in the non-dialysable portion. This fraction was concentrated to one-third of the original volume and diluted with two volumes of alcohol; the precipitate was solvent-exchanged through alcohol into benzene, was dried and weighed. Usually, however, only an aliquot of the acidified mother liquor was dialysed, and then the solids in the nondialysable portion were determined.

B. PRECIPITATION WITH ACETIC ACID

The extract was neutralized with glacial acetic acid to a pH of 6.5 to 7.0 and was concentrated at reduced pressure to a volume of one-quarter of the original. The pH was then lowered to 5 with acetic acid, and, after standing for an hour, a fine light brown precipitate slowly settled. This precipitate was collected on the centrifuge, was washed with 70% alcohol to remove any sodium acetate, was solvent-exchanged through alcohol into ether, and was then dried over phosphorus pentoxide in a vacuum desiccator. A second precipitate, obtained by adding two volumes of alcohol to the acidified mother liquor, was collected, washed and dried in the

same manner.

LARGE-SCALE EXTRACTIONS OF THE BARK WITH ALKALI

The bark, 1100 gm., with 14.05% of moisture (946 gm. moisture free), was placedin a large, stainless steel tank containing 15 litres of 2% sodium hydroxide solution. The mixture was stirred by hand for a few minutes until the bark was thoroughly wetted, and then the system was sealed and swept with nitrogen. The tank was kept under a positive nitrogen pressure during the 3-day extraction period, after which the extract was separated from the bark residue by filtration under vacuum through a Büchner funnel covered with a piece of cotton cloth. The bark residue from the extraction was poured into 6 litres of distilled water, stirred for a few minutes, and then allowed to stand overnight. The next day, the aqueous extract was filtered in the above manner, and the residue subjected to a second washing. This procedure was continued for 6 days, after which the bark was extracted with 2% alkali again. The sequence of extraction and washing was carried out three times in all. After this sequence was completed, the bark was soaked in water containing a little acetic acid to wash out the remaining alkali, was washed once more with water, was air-dried, and finally weighed. The weight on a moisture-free basis was 634 gm., or 67% of the starting material.

All the liquors were worked up in the following manner. They were neutralized to pH 6.8 to 7.0 with glacial acetic

acid, were transferred to a large, stainless steel still, heated by a steam jacket, and were concentrated at diminished pressure to a volume of about 4 litres. An additional 25 ml. of glacial acetic acid was added, and the liquor was left for a few hours (or overnight) for the precipitate to coagulate. After separation on the centrifuge, the mother liquor being stored temporarily, the precipitate was washed by stirring with 70% alcohol for a few minutes and was solvent-exchanged through alcohol into ether. Drying was over phosphorus pentoxide in a vacuum desiccator. This procedure gave fractions a-1, a-2 and a-3 from the three alkaline extracts and fractions b-1, b-2 and b-3 from the washings. Two volumes of alcohol were added to the acidified mother liquors, and the precipitate which formed was removed by centrifuging, washed with 70% alcohol, solvent-exchanged, and dried as above. The precipitates were designated as fractions c-1, c-2 and c-3 from the extracts, and d-1, d-2 and d-3 from the washings. The weights of the individual precipitates are reported in Table IV, the total being 265.2 gm., or 27.8% of the starting material, or 13.2% of the original bark.

The procedure for the second large-scale extraction was essentially the same. Four consecutive three-day extractions with the alkali were carried out with no intermediate aqueous washing. Then the residue was washed with water until nothing further was extracted. The liquors led to 14 further fractions

which are listed in Table IV. This second extraction yielded a total of 290.0 gm. of extract from 994 gm. of bark, corresponding to 29.1% of the starting material, or 13.7% of the original bark. The bark residue from the second extraction, after air-drying, was weighed, and its moisture content determined. The dry weight was 651 gm., showing that the weight loss was 343 gm., or 34.5% of the starting material.

The ash content of each of these samples was determined, and the sugars produced by acid hydrolysis were identified chromatographically. A study of the results of these analyses showed that there were two distinct fractions, one comprising the a and b samples, and the other c and d. All the a and b sub-fractions were combined to give a new Fraction A, while the c and d sub-fractions were combined into two large fractions, as previously outlined.

EXAMINATION OF THE MOTHER LIQUORS

The mother liquors from the two extractions were still highly coloured after the alcohol precipitations, and the material balance showed that about 10% of the original weight was unaccounted for. To improve this situation, the liquors were combined and concentrated to a volume of 20 litres. After the concentrate had been standing for a few days, a brown precipitate settled which was separated by decantation and centrifugation, was dried by solvent-exchange through alcohol, ether, and benzene, and then in vacuo. The resulting dark brown powder, 12.9 gm., was designated Fraction E. A

2000 ml. aliquot (10%) of the remaining liquor was then neutralized to pH 7 with sodium hydroxide solution, and dialysed in a cellophane bag against tap water for 48 hours. The non-dialysable portion was concentrated to 200 ml., and poured into an equal volume of acetone, the precipitate being collected at the centrifuge, washed with acetone and ether, and dried in vacuo. The resulting brown solid, Fraction F, weighed 2.12 gm. After further concentration, the mother liquors were poured into five volumes of acetone, but only an oily precipitate resulted. The solution was evaporated to a thick syrup at reduced pressure; in this distillation, there was a strong tendency to foam, even though Dow-Corning anti-foam compound had been added. The syrup was converted to a brown powder (Fraction G, 3.02 gm.) after drying for 48 hours at 10 mm. pressure over phosphorus pentoxide. Table V summarizes the yields and analyses of these minor fractions.

SMALL-SCALE INVESTIGATIONS OF FRACTION A

REPRECIPITATION FROM ALKALI

A sample of Fraction A weighing 5.00 gm. was stirred mechanically under a nitrogen atmosphere with 100 ml. of 2% caustic soda solution for one hour. The insoluble portion was separated on the centrifuge and was extracted for another hour with a further 25 ml. of the caustic soda. The residue from the second alkali extraction was washed twice with 25 ml. volumes of water, and was then dried, first by solvent-exchange with alcohol and ether, and then under vacuum over

phosphorus pentoxide. Drying yielded 0.82 gm. (16.4%) of alkali-insoluble material in the extract; ash, 23.8%.

The two alkali extracts and the two water washings were combined and neutralized with glacial acetic acid to pH 7. Then the solution was concentrated to a volume of 100 ml. and poured into 200 ml. of absolute ethanol. The precipitate was collected, washed with 70% ethanol to remove any sodium acetate, then with absolute ethanol and finally benzene. After being dried over phosphorus pentoxide under reduced pressure for 12 hours, the product weighed 3.93 gm. (78.6%); ash 14.5%. The total recovery was 95%. Paper chromatographic determination of the sugars produced by acid hydrolysis of these two fractions showed each to contain both glucose and uronic acid.

ATTEMPTED FRACTIONATION WITH ETHANOL

A sample of Fraction A, 1.00 gm., was shaken in a sealed bottle for 2 hours with 50 ml. of 2% sodium hydroxide. Then the mixture was acidified to pH 5 with acetic acid and the insoluble material was collected on the centrifuge. This precipitate was solvent-exchanged and dried as in the previous experiment. The weight was 0.53 gm.; ash 18.0%. Then the material remaining in solution was fractionated by collecting precipitates after the alcohol content of the solution had been brought to 33%, 50% and 80%. The precipitates were worked up in the usual manner, and the weights were 0.26, 0.04 and 0.03 gm., respectively.

TRIAL DE-ASHING

A 1.00 gm. sample of Fraction A was stirred with 150 ml. of 0.1N hydrochloric acid for 20 minutes at 5°C., after which the mixture was centrifuged and the mother liquor decanted. The de-ashed precipitate was washed with three 25 ml. volumes of distilled water. Although the pH of the third washing was still 4.8, the material was starting to become peptized, and the washing was stopped. The material was air-dried without solvent-exchange and then vacuum-dried over phosphorus pentoxide. A hard, dark brown residue remained, weighing 0.58 gm., which was ground to a powder, and found to contain 1.84% of ash. The acid solution and washings were neutralized with acetic acid and sodium hydroxide to pH 7 and were concentrated in vacuum to 35 ml. Then 100 ml. of alcohol was added, and the precipitate which formed was recovered on the centrifuge, solvent-exchanged and dried. Weight, 0.18 gm.; ash content, 22.8%.

EXTRACTION WITH AMMONIUM HYDROXIDE

A sample of 1.00 gm. of Fraction A was stirred for 4 hours in a centrifuge bottle with 50 ml. of 2% ammonium hydroxide solution. The insoluble material was separated by centrifugation, washed with water until ammonia could no longer be detected in the washings, and dried by solvent-exchange through alcohol into benzene. The residue, after drying over phosphorus pentoxide in vacuo, weighed 0.18 gm. The ammonia-soluble portion of Fraction A was precipitated

from the combined extract and aqueous washings by the addition of two volumes of alcohol, was redissolved in 50 ml. of water, and was reprecipitated by the addition of alcoholic hydrochloric acid until a pH of 1 was reached. The precipitate was washed with distilled water until the washings were neutral, and then with ethanol, and finally benzene. After being dried in vacuo, the residual solid weighed 0.84 gm. The liquors from both precipitations of the extract were very dark in colour, and no doubt contained further material so that the final recovery would have been well over 100%.

EXTRACTION WITH AMMONIUM OXALATE

A portion of Fraction A, 10.0 gm., was stirred at 80° with 100 ml. of 2% ammonium oxalate for 2 hours. Then the insoluble material was centrifuged from the solution and again extracted with 100 ml. of the ammonium oxalate solution for 2 hours. The residue was washed successively with water, alcohol, acetone and ether and was then dried, yielding 2.35 gm. of a light brown powder. The two extracts were combined and precipitated by addition of 500 ml. of ethanol. The precipitate was collected on the centrifuge, washed with 70% alcohol, solvent-exchanged through alcohol and acetone into ether and dried over phosphorus pentoxide. Yield, 7.73 gm.; the mother liquors were highly coloured, but were not examined further.

ATTEMPTED HYDROLYSIS OF FRACTION A WITH EMULSIN

The activity of the emulsin, supplied by Nutritional Biochemical Corporation, was confirmed by an experiment using salicin. This glucoside showed an increase in copper reducing power equivalent to 80% of the theoretical amount of glucose after treatment with the emulsin for 12 hours. A sample, 0.100 gm., of Fraction A was stirred with 5 ml. of 2% sodium hydroxide solution for one hour. Then 25 ml. of water was added and the insoluble material was separated by centrifugation. The pH of the solution was adjusted to 5.0 with 10% acetic acid, and then diluted to 50 ml. in a volumetric flask. Then 5 mgm. of emulsin was added, and a 5 ml. sample was taken and its copper reducing power determined. Samples were also removed after 6 and 16 hours, and analysed. The volumes of 0.005N sodium thiosulphate solutions consumed in these analyses were 8.34, 8.38 and 8.36 ml. respectively, or were constant to within experimental error. These values correspond to 1.23 mgm. of glucose per 5 ml. of solution, or per 10 mgm. of the original Fraction A. To show that the activity of the emulsin was not being destroyed in the medium, a parallel experiment was conducted using a mixture of 0.100 gm. of Fraction A and 0.025 gm. of salicin. In this experiment the increase in copper reducing power indicated that 69% of the theoretical amount of glucose had been produced. The high reducing power of Fraction A before hydrolysis was probably caused by non-carbohydrate constituents.

ACETYLATION OF FRACTION A

Since test tube experiments had shown that this extract was swollen in formamide, but unaffected by pyridine, the method of Carson and Maclay (106) was used for acetylation. A sample weighing 1.00 gm. was stirred for 4 hours with formamide; although the material was not very highly swollen, no further swelling appeared to be taking place. Then 20 ml. of anhydrous pyridine was added and the mixture was stirred for another 30 minutes. Ten ml. of re-distilled acetic anhydride was then introduced to the stirred suspension through a dropping funnel over a period of 30 minutes, and the reaction mixture was stirred at room temperature for 48 hours. The insoluble material was separated with the centrifuge and was washed three times with water, then alcohol, and finally benzene, and was then dried under vacuum. Yield, 0.41 gm.; acetyl, 23.0%. The soluble portion of the reaction mixture was precipitated by pouring onto 50 gm. of crushed ice mixed with 100 ml. of water, and the precipitate was collected, washed and dried as above. Weight, 0.26 gm.; acetyl, 27.6%. These recoveries only accounted for 51% of the starting material. A considerable amount of material appeared to be dispersed in the aqueous liquors, and could not be precipitated by the addition of ethanol. A precipitate formed when these liquors were acidified with hydrochloric acid, but after washing, solvent-exchange, and drying, only accounted for 0.04 gm. of the original extract.

REACTION OF FRACTION A WITH SODIUM CHLORITE

A portion of Fraction A, 10 gm., was stirred with 250 ml. of distilled water until it was completely suspended, and the pH was adjusted to 4.8 with glacial acetic acid. Then 16.7 gm. of technical sodium chlorite was added in small portions over a period of 2 hours; the mixture was stirred throughout, and the pH was maintained at 4.8 by the addition of acetic acid as required. The temperature was kept below 30° by means of a water bath. After about 4 hours, the mixture became quite light in colour. After being stirred for two more hours, the insoluble material was collected on the centrifuge, and was washed with 50 ml. volumes of 70% alcohol until the sodium chlorite was completely removed, as shown by its failure to give a colour to acidified potassium iodide-starch solution. Six washings were required in all. The residue was dried, first by solvent-exchange through alcohol, acetone, and ether, and finally at reduced pressure overnight over phosphorus pentoxide. Yield, 3.95 gm.

When the mother liquor was poured into two volumes of ethanol, a sticky mass settled. This mass was taken up in 100 ml. of water, and an equal volume of alcohol was added. The precipitate, a light brown solid, was recovered by centrifugation, and dried, first by solvent-exchange, and then in a vacuum desiccator. Yield, 1.05 gm.

A sample, 1.00 gm., of the water-insoluble product was given a second treatment with 1.00 gm. of sodium chlorite in 25 ml. of water. After the reaction mixture had been worked

up in the above manner, a water-insoluble product weighing 0.83 gm., and a water-soluble substance weighing 0.07 gm., were obtained.

LARGE-SCALE BLEACHING OF FRACTION A

The procedure was a modification of that used in the smaller-scale experiments. Bleaching was carried out in three batches, 40 gm. of Fraction A being used in each case. This amount was dissolved by stirring with 400 ml. of 2% caustic solution for one hour. Then 400 ml. of water was added and the solution was neutralized to pH 4.8 with glacial acetic acid. The sodium acetate-acetic acid medium formed during this treatment acted as a buffer, and the pH remained constant at this value throughout the experiment. Over a period of two hours, 50 gm. of technical sodium chlorite was added to the stirred suspension, the reaction temperature being kept below 30° by the addition of a little ice when necessary. The products from the three runs were collected and treated as before, and weighed 19.4, 17.5 and 19.3 gm. Some material was lost in the second experiment because of foaming. The total yield was 56.2 gm., or 46.8% of the starting material. This water-insoluble, bleached material was hereafter denoted as Fraction A-1. A second, water-soluble portion, Fraction A-2 was obtained when the three combined mother liquors were poured into an equal volume of alcohol. This fraction, when washed and dried, amounted to 12.9 gm. (10.8%) of a light tan powder. The total

recovery of starting material was 57.6%. These materials were analysed for carbohydrates, as summarized in Table VII.

SMALL-SCALE INVESTIGATION OF FRACTION A-1

DEGREE OF HYDROLYSIS

Samples of about 0.025 gm. were accurately weighed into glass tubes, 1 ml. of N sulphuric acid was added, and the glass tubes were sealed. The samples were then placed in an oven at 95° for 6 hours, with occasional shaking. Then the tubes were opened, the contents completely transferred with washing to a volumetric flask, diluted to 50 ml. with water, and 5 ml. aliquots were analysed for their copper reducing power. The degree of hydrolysis of Fractions A, A-1 and A-2 was determined in this manner, giving the values listed in Table VII.

OXIDATION AND HYDROLYSIS TO MUCIC ACID

A sample of Fraction A-1, 0.50 gm., was placed in a round-bottom flask and 50 ml. of N hydrobromic acid containing 0.5 ml. of bromine was added. The flask was fitted with a reflux condenser and an electric heating mantle, and was heated at reflux temperature for 24 hours. A few drops of bromine were added through the top of the condenser from time to time as the liquid became colourless. The insoluble material was removed by filtration through glass wool; the filtrate was concentrated to a volume of 10 ml. at reduced pressure and allowed to stand at 5° overnight. A white,

crystalline solid formed, which was collected by filtration, dried and weighed. Yield, 0.10 gm. This product was twice recrystallized from boiling water to yield 0.040 gm. of colourless crystals, melting at 210-211° with decomposition. This melting point was undepressed when mixed with an authentic sample of mucic acid. The filtrate from the mucic acid crystals was neutralized with potassium hydroxide, evaporated to dryness, and 2 ml. of water was added to the residue. This residue was then acidifed with hydrochloric acid, extracted with 10 ml. of alcohol, and the extract evaporated to dryness. The residue was neutralized with potassium carbonate, 1 c.c. of glacial acetic acid was added, and the mixture left to stand for several weeks. No crystals of potassium acid saccharate were formed during this time.

ACETYLATION

A. With Sodium Acetate

A mixture of 2.5 ml. of redistilled acetic anhydride and 0.12 gm. of fused sodium acetate was heated in a water bath at 90°; then 0.50 gm. of Fraction A-1 was added in portions over a period of about 15 minutes. The mixture was heated on a steam bath for 4 hours, and shaken thoroughly at regular intervals. Then a vacuum was applied to the system, and the excess acetic anhydride was distilled off *in vacuo*. The residual paste was drowned in 25 ml. of ice water and the precipitate which formed was collected, washed five times with water, then with alcohol and benzene, and

finally dried. Yield, 0.18 gm.; acetyl, 28.2%.

The mother liquors from the precipitation were concentrated at reduced pressure to one-third of their volume and were poured into 100 ml. of ethanol. The precipitate was treated in the above manner, yielding 0.11 gm. of dark brown product with 20.9% of acetyl groups.

B. With Pyridine-Formamide

A mixture of 3.0 gm. of Fraction A-1 and 60 ml. of formamide was stirred at 40° for about an hour, in order to convert the solid to a highly swollen gelatinous mass. Then 30 ml. of pyridine (distilled from barium oxide) was added, the mixture was stirred for 2 hours more, and was cooled to room temperature. Redistilled acetic anhydride, 30 ml., was then added through a dropping funnel, the mixture was stirred at room temperature for 24 hours, was heated to 40° and stirred at this temperature for another 2 hours. Then the acetate was precipitated by pouring the mixture onto a mixture of 100 gm. of ice and 200 ml. of water. After standing for an hour at 5°, the insoluble material was removed on the centrifuge, was washed three times with water, twice with 70% ethanol, and three times with absolute ethanol. When the product was washed with benzene, part of it dissolved, but was reprecipitated by the addition of an equal volume of petroleum ether and was thereafter treated separately. The benzene-insoluble material was washed once more with benzene and dried over phosphorus pentoxide and paraffin at reduced press-

ure. Yield, 0.55 gm.; acetyl, 25.5% (Acetate I). The benzene-soluble portion was washed twice with petroleum ether and dried in the same manner. Yield, 0.54 gm.; acetyl, 40.7% (Acetate II). These two fractions only accounted for about one-quarter of the original material, after allowing for their acetyl content.

The aqueous liquor from the acetylation was concentrated in vacuo to a volume of 80 ml., and 150 ml. of ethanol was added. The precipitate coagulated only very slowly, and was left to stand at 5° overnight. After being recovered on the centrifuge, exchanged through 70% ethanol, absolute ethanol, and benzene, and finally dried, as above, the yield was 1.01 gm.; acetyl, 23.2% (Acetate III). The mother liquors from this precipitation were concentrated to 50 ml. and poured into 10 volumes of alcohol. The precipitate was recovered as above. Yield, 0.39 gm.; acetyl, 28.6% (Acetate IV). These four precipitates, when corrected for acetyl content, only accounted for 60% of the original Fraction A-1.

A sample of Acetate III, 0.50 gm., was reacetylated by the same method, using 10 ml. of formamide, 20 ml. of pyridine, and 20 ml. of acetic anhydride. When the mixture was poured onto ice water, only a negligible quantity of material precipitated, and the solution was evaporated to 40 ml., and poured into two volumes of ethanol. The precipitate was collected, washed with 70% ethanol, absolute ethanol, and benzene, and dried. Yield, 0.42 gm.; acetyl, 32.2%.

Small samples of Acetates I, II, and III were deacetylated by immersing them first in alcoholic potassium hydroxide for 2 hours, and then overnight in aqueous alkali. The materials recovered from these reactions were each hydrolysed with sulphuric acid, and then the sugars were studied by paper partition chromatography. Acetate I yielded strong spots corresponding to glucose and uronic acid, and much weaker spots of xylose and arabinose. Acetate II showed very strong glucose spots and no other sugar or uronic acid, while Acetate III showed only the presence or uronic acid.

EXTRACTION OF FRACTION A-1 WITH ALKALI

A sample of Fraction A-1, 5.00 gm., was stirred with 100 ml. of 2% sodium hydroxide solution for one hour and the insoluble portion was recovered at the centrifuge. This residue was washed for another hour with 50 ml. of the alkali, then twice with 25 ml. volumes of distilled water. Since the washings appeared to contain a considerable amount of dispersed material, they were combined with the alkaline extract. The undissolved material was washed with ethanol and benzene and then dried. Yield, 1.43 gm. (28.6%). The extract and washings were neutralized to pH 6 with acetic acid and poured into 2 volumes of ethanol. The precipitate was collected at the centrifuge, washed with alcohol, acetone and ether, and dried. Yield, 3.22 g. (64.4%). Total recovery, 92.0%.

PRECIPITATION OF THE ALKALINE EXTRACT WITH ACID

A 1.0 gm. sample of Fraction A-1 (ash, 21.8%) was extracted with alkali and water as in the previous experiment. This extract was acidified with 10% sulphuric acid until the pH was less than 1. The precipitate was collected, washed with water, alcohol, and ether, and dried. Yield, 0.26 gm. (ash, 6.05%). The mother liquor was neutralized to pH 6 with sodium hydroxide solution and then dialysed against running water in a cellophane membrane for 36 hours. A precipitate which settled during this time was collected and dried. Weight, 0.19 gm. (ash, 13.3%). The remainder of the extract was precipitated by pouring the non-dialysable solution into 500 ml. of ethanol. The dried precipitate weighed 0.15 gm. Samples of each of these three products were hydrolysed and the free sugars separated chromatographically. All three hydrolysates showed spots of glucose and uronic acid, of about the same strength, and it appeared that little fractionation had occurred during this separation.

PRECIPITATION OF CALCIUM PECTATE FROM THE ALKALINE EXTRACT

The alkaline solution, prepared as in the previous experiment, was neutralized to pH 6 with acetic acid. A small precipitate which formed was recovered, solvent-exchanged, and dried. Yield, 0.15 gm. The neutralized mother liquor was then mixed with 10 ml. of 10% calcium chloride solution, and the precipitate was separated, washed with water, alcohol and ether, and dried, yielding 0.42 gm. of a slightly yellow

powder containing 11.7% of ash. The liquors were dialysed as before and precipitated with ethanol but yielded only 0.07 gm. Samples of these three fractions were also hydrolysed and their sugars identified chromatographically. It appeared that a better separation was obtained in this case, as the uronic acid predominated in the second precipitate with calcium chloride, while the first and third precipitates contained the bulk of the glucose.

ACETYLATION OF THE RESIDUE FROM THE ALKALINE EXTRACTION

A 1.0 gm. sample of the portion of Fraction A-1 not dissolved in alkali was acetylated at 45°C. for 24 hours, using 5 ml. of formamide, 10 ml. of pyridine and 10 ml. of acetic anhydride. The product isolated by pouring the reaction mixture onto ice weighed 0.58 gm. and contained only 5.6% of acetyl. Another fraction was isolated when the mother liquor was concentrated to 50 ml. and poured into 150 ml. of ethanol. This portion, 0.21 gm., contained 11.5% of acetyl group.

ACETYLATION OF THE ALKALINE EXTRACT

A sample of the material precipitated from the above alkaline extract, weighing 3.00 gm., was swollen in formamide by stirring for 2 hours at 45°. Then 20 ml. of pyridine was added, the mixture was cooled to room temperature and 20 ml. of acetic anhydride was added dropwise. The reaction mixture was stirred at 45° for 24 hours, and then poured into 300 ml.

of ice water and allowed to stand at 5° for 4 hours. The precipitate was separated, washed with water, alcohol, and petroleum ether, and dried. Yield, 0.26 gm.; acetyl 40.9%. The material dispersed in the water was recovered in two portions. The first was isolated by concentrating to 100 ml. and pouring into 200 ml. of ethanol. Yield, 1.13 gm.; acetyl, 28.6%. The second was obtained by evaporating to 20 ml. and then adding 100 ml. of ethanol. Weight, 0.82 gm.; acetyl, 24.3%.

LARGE-SCALE EXTRACTION OF FRACTION A-1 WITH ALKALI

Fraction A-1, 60.0 gm., was stirred with 400 ml. of 2% sodium hydroxide solution for 4 hours under a nitrogen atmosphere. The insoluble material was separated on the centrifuge and extracted again with 200 ml. of the alkali. The two extracts were combined. The insoluble residue was washed once with 100 ml. of 1% hydrochloric acid which had been cooled to 0°; then twice with 50 ml. volumes of water. These aqueous washings appeared to contain a considerable amount of dispersed material, and they were treated separately. The residue was dried by solvent-exchange with alcohol and ether, and then *in vacuo*. Weight, 23.7 gm.; uronic anhydride, 38.3% (Fraction A-1a). A second precipitate was obtained by the addition of two volumes of ethanol to the aqueous washings, solvent-exchanging, and drying. Yield, 5.83 gm.; uronic anhydride, 94.0% (Fraction A-1b).

The alkaline extracts were acidified to pH 4.5 with glac-

ial acetic acid, and were then diluted by the addition of 2 volumes of alcohol. The precipitate was also washed with 50 ml. of cold, 1% hydrochloric acid, then with water, was solvent-exchanged with alcohol and ether, and dried. Yield, 12.5 gm.; uronic anhydride, 29.7% (Fraction A-1e). A further precipitate weighing 3.63 gm. was recovered from the aqueous washings (Fraction A-1f). This sub-fraction had 55.5% of uronic anhydride.

The pectic material remaining in the Fraction A-1a was extracted by allowing it to disperse into water. This fraction, 20.0 gm., was stirred vigorously for 2 hours with three successive volumes of water, was recovered, solvent-exchanged, and dried. Weight, 12.67 gm.; uronic anhydride, 5.28% (Fraction A-1c). Each of the three extracts was diluted with ethanol, the precipitates solvent-exchanged through ethanol, acetone and ether, and dried in vacuo. The yields were 5.52 gm., 1.75 gm., and 0.15 gm. The first two were combined (Fraction A-1d) and an analysis for uronic anhydride content gave 93.8%. On this basis this material was combined with Fraction A-1b.

ISOLATION OF PECTIC ACID FROM THE CALCIUM PECTATE

A sample of the pectin isolated in the previous experiment was ashed at 650°C.; ash, 11.3%. A test of the acid-soluble portion of the ash with ammonium oxalate solution showed there was a considerable amount of calcium present, indicating that the original pectin was in the form of calcium

pectate. The original bark and Fractions A and A-1 also gave positive tests for calcium when treated in this manner.

A sample of the calcium pectate, 2.00 gm., was extracted twice at room temperature with 20 ml. of ammonium oxalate solution. The residue was washed with water and dried at reduced pressure. Weight, 0.14 gm. The two extracts were precipitated with ethanol, the precipitate redissolved in 50 ml. of water, and reprecipitated by the addition of 100 ml. of ethanol containing 5 ml. of concentrated hydrochloric acid. The precipitate was collected at the centrifuge, washed with 70% ethanol, absolute ethanol, and ether, and dried under high vacuum over phosphorus pentoxide for 24 hours. Yield, 1.82 gm. An attempt to measure the rotation of this pectic acid was unsuccessful because of the turbidity of its solutions in either ammonium or sodium hydroxide.

PURIFICATION OF THE PECTIC ACID

A sample of the impure pectic acid weighing 1.60 gm. was dissolved in 25 ml. of 2% ammonium hydroxide solution and was precipitated by the addition of 5 ml. of 10% calcium chloride solution. The precipitate was recovered, washed twice with water and then redissolved by stirring for a few minutes with 50 ml. of 2% ammonium oxalate solution. This cycle of precipitation by calcium chloride followed by dissolution in ammonium oxalate was carried out 5 times. Then the ammonium oxalate solution was dialysed against running water in a cellophane bag for 24 hours, and the free pectic acid was

reprecipitated with alcohol containing a little hydrochloric acid. The precipitate was redissolved in ammonium hydroxide, reprecipitated with acidified alcohol, and washed with alcohol, acetone, and ether. In spite of this washing, the pectic acid was still in the form of a wet gel. This product was dried in vacuo over phosphorus pentoxide to a hard, white mass, which was ground to a powder in a mortar and dried at a pressure of 0.01 mm. of mercury for 24 hours. Yield, 0.97 gm.; ash, 0.52%; uronic anhydride, 94.7%; $[\alpha]_D^{25}$ =+265° (c = 0.7 in 1% ammonium hydroxide).

FORMATION OF MUCIC ACID FROM THE CALCIUM PECTATE

Since the yield of mucic acid in the earlier oxidation-hydrolysis only accounted for a small portion of the total uronic acid, a similar experiment was conducted on the calcium pectate. In this case, a 0.50 gm. sample was mixed with 50 ml. of N hydrobromic acid and 1 ml. of bromine and left, with occasional shaking, for 3 days. Then the mixture was heated under reflux, and the mucic acid recovered as in the earlier experiment. This procedure yielded 0.34 gm. of mucic acid, melting at 212-213° (corrected) with decomposition, or 65% of the theoretical yield.

ACETYLATION OF THE FRACTION A-1e

Technique was first tested on a 1 gm. sample, using the formamide method. As in the earlier case, three fractions were obtained, one benzene-soluble, one benzene-insoluble and

a third dispersed in the water.

The remaining alkali-soluble fraction, 11.0 gm., was stirred with 100 ml. of formamide (redistilled) at 40° for 1 hour, and then 150 ml. of anhydrous pyridine was added, and the mixture stirred at 40°C. for 2 hours. The heating bath was then removed and 100 ml. of distilled acetic anhydride was added dropwise over a period of 1 hour. After this mixture was stirred at 40° for 24 hours, it was poured into 1 litre of water containing 500 gm. of ice. The mixture was allowed to stand overnight at 5°C., as the precipitate appeared to coagulate slowly. The precipitate was recovered, washed three times with water, three times with ethanol, and stirred three times with 50 ml. volumes of benzene before being dried. Yield, 5.0 gm.; acetyl, 40.6% (Acetate Ie). The benzene extracts were poured into 200 ml. of petroleum ether, the precipitate washed with petroleum ether, and dried. Yield, 6.17 gm.; acetyl, 43.9%; uronic anhydride, 2.75% (Acetate IIe). This acetate gave a somewhat coloured solution in chloroform, but it was sufficiently transparent to permit accurate readings on the polarimeter; $[\alpha]_D^{25} = +178°$ (c. = 0.38 in chloroform).

The aqueous mother liquors from the precipitation were concentrated to 300 ml. and poured into 600 ml. of ethanol. The precipitate was separated, washed with ethanol and benzene, and dried. Yield, 2.63 gm.; acetyl, 23.1% (Acetate IIIe). A second precipitate was recovered by evaporation to 125 ml.

and the addition of 500 ml. of ethanol. Yield, 1.87 gm.; acetyl, 19.8% (Acetate IVe). These two were combined and the uronic anhydride content was found to be 43.4%, showing them to be largely pectin acetates.

The benzene-insoluble acetate from the above experiment was reacetylated by swelling in pyridine (50 ml.) for 4 hours at 50° and by adding 20 ml. of acetic anhydride slowly at room temperature. The mixture was stirred for 12 hours at this temperature and for 12 hours at 45°. After being recovered in the same manner as before, the product was separated into a benzene-insoluble acetate, 2.29 gm., and containing 39.8% of acetyl, and a benzene-soluble acetate weighing 2.19 gm. and having 42.7% of acetyl groups; this product was combined with the previous fraction which had been extracted by benzene. No attempt was made to recover the small amount of material dispersed in the water.

As the acetyl value of this starch acetate was a little less than the theoretical value of 44.8%, another acetylation was attempted. A portion of 5.00 gm. was swollen in 50 ml. of anhydrous pyridine, then 15 ml. of formamide was added and finally 20 ml. of redistilled acetic anhydride through a dropping funnel. The reaction was carried out at room temperature for 12 hours and then at 40° for 6 hours. The mixture was poured into a beaker containing 250 gm. of ice and 400 ml. of water, and one hour later the precipitate was collected, washed with water and alcohol and then with benzene.

The benzene extract was precipitated with petroleum ether and dried. Yield, 4.08 gm. The acetyl content remained unchanged at 43.4% during this reaction, but the uronic anhydride content had fallen from 2.75% to 1.19%. A benzene-insoluble acetate was also recovered, weighing 0.47 gm., and containing 39.6% of acetyl groups. The pectin acetate in the mother liquor was not isolated.

ACETYLATION OF FRACTION A-1f

A 3.0 gm. sample of Fraction A-1f was swollen in 25 ml. of formamide over a one hour period, was then heated to 40°, and diluted with 50 ml. of pyridine. After being stirred for two more hours, the mixture was cooled to room temperature and 30 ml. of acetic anhydride was added in portions over a one hour period; the suspension was stirred at this temperature for 12 hours, then at 40° for 12 hours. Then the mixture was poured onto 300 ml. of water and 150 gm. of ice, the precipitate washed with water, ethanol and benzene, and dried *in vacuo*. Yield, 0.57 gm.; acetyl, 32.2%. None of the product was lost by solution in the benzene washings. The mother liquor was concentrated to 150 ml. and poured into 300 ml. of ethanol. The precipitate was washed and dried as before. Yield, 1.64 gm.; acetyl, 19.5%.

DEACETYLATION OF THE PECTIN ACETATE

A 2.0 gm. sample of the acetate recovered from the aqueous mother liquor from the previous acetylation (Acetate IIIe) was placed in a three-neck flask fitted with a nitrogen inlet, a

mechanical stirrer, and a dropping funnel. Then 20 ml. of acetone was added, and the mixture was stirred for about 1 hour. A part of the material dissolved, and the remainder became highly swollen. The system was swept thoroughly with nitrogen, and cooled to 0°, and 25 ml. of N sodium hydroxide solution was added dropwise. The mixture was allowed to warm up to room temperature, and was stirred under a constant flow of nitrogen for 12 hours. Then the product was recovered by acidification to pH 2 with 5% hydrochloric acid, followed by the addition of 2 volumes of alcohol. The liquid was removed at the centrifuge, and the pectin redissolved by extracting the precipitate three times with 25 ml. volumes of ammonium oxalate solution; reprecipitation as the calcium salt and re-solution in ammonium oxalate was repeated three times. The final solution was dialysed against water for 48 hours and the non-dialysable solution was precipitated by pouring into 250 ml. of ethanol containing 5 ml. of concentrated hydrochloric acid. The precipitate was redissolved in ammonia, filtered, reprecipitated, washed four times with alcohol, three times with acetone and three times with benzene. After the residue had been dried under high vacuum for 24 hours, the yield was 0.67 gm., or 78% of the theoretical, based on the uronic anhydride content of the original material. Ash, 1.33%; uronic anhydride, 84.8%; $[\alpha]_D^{22}$ = +226° (c = 0.24 in 2% ammonia).

DEACETYLATION OF THE STARCH ACETATE

A sample of the benzene-soluble acetate (Acetate IIe) was dissolved in 75 ml. of dioxane, and the reaction flask was thoroughly swept with nitrogen. After adding 75 ml. of N alcoholic potassium hydroxide, the reaction mixture was stirred under nitrogen for 4 hours, the precipitate which formed during this time being recovered on the centrifuge and returned to the reaction flask. The flask was swept again with nitrogen, and then 50 ml. of N sodium hydroxide solution was added. The mixture was stirred overnight under nitrogen, then neutralized with glacial acetic acid, and the starch precipitated with ethanol. After being recovered at the centrifuge, the product was washed with 70% ethanol, and dried, first by solvent-exchange with ethanol, acetone, and ether, and finally in vacuo over phosphorus pentoxide. Yield, 1.88 gm. However, this product still contained more than 3% of acetyl groups, and the deacetylation with aqueous alkali was accordingly repeated. This time, the product, weighing 1.62 gm. (94%) had a negligible acetyl content. Ash, 1.10%; uronic anhydride, 2.90%; $[\alpha]_D^{22} = +139.5^\circ$ (c = 0.16 in 1% sodium hydroxide solution).

HYDROLYSIS OF THE STARCH BY ACID

A sample of the deacetylated starch, 0.19 gm., was made into a paste with a few drops of water, and then 20 ml. of N sulphuric acid was added. The flask was fitted with a reflux condenser and heated at 90° for 12 hours, then at 100° for 3

hours more. The insoluble material was recovered at the centrifuge, washed twice with water, then twice with alcohol, and dried under reduced pressure. Yield, 0.015 gm. The acidic solution was passed twice through an Amberlite IR-4B anion exchange column, after which the pH was found to be 6.3. Evaporation of the effluent under diminished pressure left a clear, light brown oil, which did not crystallize after drying under high vacuum. A solution of this oil in 4 ml. of water was mixed with 0.4 gm. of phenylhydrazine hydrochloride and 0.6 gm. of sodium acetate, and placed in a boiling water bath for 20 minutes. The yellow precipitate of glucosazone (124) which formed was recovered, washed with a little water, and air-dried. To form the osotriazole the crystals were boiled for one hour under reflux with 5 ml. of water, 0.25 ml. of 0.5N sulphuric acid, 0.15 gm. of copper sulphate pentahydrate, and 3 ml. of isopropyl alcohol. The resulting solution was concentrated to 3 ml. under a stream of air on a steam bath and then cooled to 5°C. The crystals which separated were washed with water, and dissolved in 5 ml. of boiling water. A little Darco was added, the solution was boiled for a few minutes, and filtered while still hot. After the solution had been allowed to stand at 5° overnight, the colourless, long needles were separated and dried. Yield, 0.015 gm., melting point, 194.5-196°. This melting point was undepressed when the product was mixed with a sample of glucosotriazole prepared from glucose (125) by the same method.

ENZYMATIC HYDROLYSIS OF THE STARCH

A 0.01 gm. sample of the starch was made into a paste with 2 drops of water, and then 10 ml. of boiling water was added. Most of the solid went into solution, while the rest became highly swollen. Then 5 ml. of a phosphate buffer solution (126) was added, bringing the pH to 5.3. The mixture was heated to 75° in a water bath, and 0.01 gm. of amylase (Nutrition Biochemical Corp.) was added. The mixture was maintained at this temperature with occasional stirring, for 1 hour, before being cooled to 50°; another 0.01 gm. of the amylase was added, and the solution was kept at this temperature for an hour. After having been kept at 35° for 12 hours, the solution no longer gave any colouration with iodine, whereas the original had given the deep blue colour characteristic of starch.

Two volumes of alcohol were now added to the solution, and the insoluble material was separated at the centrifuge. The mother liquor was evaporated to dryness under reduced pressure, the residue was extracted with 2 ml. of warm, 70% ethanol, and the extract was evaporated to dryness. This residue was taken up in 0.1 ml. of water, and a 0.01 ml. sample of this solution was studied by paper partition chromatography. Three spots appeared on the developed and sprayed paper - a strong one identical in mobility with maltose, a weak one corresponding to glucose, and a strong third one, which was not identified, but which had a mobility exactly half that of maltose.

INVESTIGATION OF FRACTION C

BLEACHING WITH SODIUM CHLORITE

A sample of Fraction C weighing 3.0 gm. was stirred with 50 ml. of distilled water for 15 minutes, and the pH of the solution was adjusted to 4.8 with 10% acetic acid. Then 3.0 gm. of technical sodium chlorite was added to the stirred solution in portions over a period of 2 hours, the pH being maintained at its initial value by the addition of acetic acid as required. After the addition of the sodium chlorite was completed, the stirring was continued for 4 hours. Towards the end of this period, the mixture became noticeably lighter in colour, and the temperature rose to 30°. The insoluble material was collected at the centrifuge and washed with 70% ethanol until the washings gave a negative test for sodium chlorite with acidified potassium iodide-starch reagent. Then the residue was dried by solvent-exchange with alcohol, acetone and ether, and finally in a vacuum desiccator, yielding 0.23 gm. of Fraction C-1, a cream-coloured precipitate containing 24.5% of ash. The mother liquor from the extraction was poured into two volumes of ethanol and the precipitate collected at the centrifuge, washed with 70% ethanol, absolute ethanol, acetone, and ether, and dried in the same manner as the water-insoluble precipitate. This treatment yielded 2.04 gm. of a slightly yellow powder, Fraction C-2, containing 10.0% of ash.

ENZYMATIC HYDROLYSIS OF FRACTION C-2

A 0.2 gm. portion of Fraction C-2 was made into a paste with a few drops of water, and the paste was thoroughly dispersed by stirring in 10 ml. of hot water. Following Pigman's directions (126), the solution was heated to 75°, and 0.01 gm. of alpha amylase was added. A few drops of the solution at this point imparted a purple colour to a dilute solution of iodine in aqueous potassium iodide. After the addition of 5 ml. of a phosphate solution buffered at pH 5.2, the solution was stirred at 75° for one hour, then cooled to 50°. Another 0.01 gm. of amylase was added and stirring continued for an hour. Finally, the mixture was cooled to 30°, another 0.01 gm. of amylase was added, and the solution stirred overnight at this temperature. Next day, the mixture, which now gave no colour with iodine, was diluted with three volumes of alcohol, and the precipitate was collected at the centrifuge. After dialysis to remove the inorganic material, a sample was hydrolysed with sulphuric acid and the sugars separated by chromatography. Comparison with a hydrolysed sample of the untreated Fraction C-2 showed that there was a slight weakening of the glucose spot. The alcoholic liquor was evaporated to dryness *in vacuo*, the residue extracted with 5 ml. of hot, 75% ethanol and the extract evaporated to dryness. The paper partition chromatogram of the residue indicated the same three spots as were found after the enzymatic hydrolysis of the starch isolated from Fraction A.

EXAMINATION OF THE BARK RESIDUE

EXHAUSTIVE EXTRACTION OF THE BARK WITH ALKALI

A sample of the bark residue from the large-scale extractions with alkali was ground to pass through a 40-mesh sieve in a Wiley mill, and a 70.0 gm. sample (6.76% moisture; dry weight 65.3 gm.) was extracted with 900 ml. of 10% caustic soda under a nitrogen atmosphere for 3 days at room temperature with vigorous stirring. The liquor was separated on the centrifuge and the treatment repeated. The two liquors were treated in the usual manner to yield 3.98 gm. of water-insoluble precipitate and 7.14 gm. of the alcohol-insoluble precipitate. The bark was washed with 200 ml. volumes of water until the washings were nearly colourless, 10 washings being required. These washings, worked up in the usual manner, yielded 1.46 gm. of the water-insoluble fraction and 3.08 gm. of the alcohol-insoluble material. The bark residue was washed with 200 ml. of 1% acetic acid, twice with water, twice with ethanol, and then dried *in vacuo* for 48 hours. This procedure yielded 50.9 gm. of product containing 4.59% of moisture (dry weight, 48.6 gm.), or 74.4% of the starting material.

CHLORITE BLEACHING OF THE EXHAUSTIVELY-EXTRACTED BARK

A 21.0 gm. sample of the exhaustively-extracted bark (dry weight, 20.1 gm.) was stirred with 1 litre of water until it had become thoroughly wetted, and 10 gm. of sodium acetate was dissolved in the suspension. Acetic acid was used to adjust

the pH to 4.5, and sodium chlorite (35.0 gm.) was added over a period of an hour. When this addition was complete, the reaction mixture was placed in a bath at 50°C., and stirred at this temperature for 8 hours. Then the liquid was removed on a sintered glass crucible, and the residual bark was washed with water until the washings came through free of chlorite. The residue was washed with alcohol, and dried at reduced pressure over phosphorus pentoxide for 24 hours. Yield, 16.2 gm. (15.9 gm. dry weight, 78.9% of the starting weight).

EXTRACTION OF THE BLEACHED BARK WITH AQUEOUS AMMONIA

Anderson (83) reported that a part of the pectic material in the various woods he studied could only be extracted by ammonium hydroxide after chlorination. An attempt was made to extract the remaining 3.03% of uronic acid in the bleached bark residue by this solvent. A 6.0 gm. portion (dry weight 5.86 gm.) was stirred for 4 hours at room temperature with 200 ml. of 2% aqueous ammonia, and then twice with 50 ml. volumes for 1 hour periods. The bark was washed with water and ethanol, and dried *in vacuo* overnight. Yield, 5.62 gm. (dry weight, 5.39 gm.). The ammonia extract and washings were combined and neutralized to pH 6 with acetic acid. Then 10 ml. of 10% calcium chloride solution was added. A small precipitate was formed, which was collected at the centrifuge, washed with water, ethanol, and ether, and dried. Yield, 0.12 gm. A further small precipitate was formed on addition of ethanol, but was not recovered. The residual bark still showed 2.04% of uronic

anhydride.

IDENTIFICATION OF GLUCOSE IN THE BARK HYDROLYSATE

A 100 ml. portion of the mother liquors from the lignin determination on the bleached bark residue was warmed to 60°C. and was treated with solid barium carbonate until the liquid was red to congo red paper. The barium sulphate was separated at the centrifuge, washed with a little water, and the combined liquor and washings were evaporated to dryness at reduced pressure. The residual syrup was taken up in 4 ml. of water and converted to glucosotriazole by the method outlined above. Yield, 0.028 gm. Melting point, 195.5-196°, undepressed when mixed with an authentic sample.

SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

1. The extraction by alkali of white spruce bark which had previously been extracted in succession with methanol, water, liquid ammonia, and again with water was studied on a small scale under various conditions. These extractions indicated that a large portion of this material was soluble in dilute solutions of sodium hydroxide even when the process was carried out under very mild conditions.

2. In applying the results of these preliminary experiments, two extractions were carried out, each on one kilogram of the residual white spruce bark, using two per cent sodium hydroxide at room temperature in a nitrogen atmosphere. The extractions produced a weight loss of 33.6 per cent in the starting material. Three major and three minor fractions totalling 31.6 per cent of the starting material, or 14.5 per cent of the original bark, were recovered from the extracts. The largest of these, Fraction A, was a dark, water-insoluble amorphous solid, amounting to seven point eight per cent of the original bark, while there were two large water-soluble fractions, Fraction C, two point ninety-three per cent, and Fraction D, two point sixty-nine per cent.

3. Fraction A was bleached with sodium chlorite at pH four point seven and room temperature. A yield of 46.8 per cent of a light-coloured, water-insoluble residue resulted, while a further ten point eight per cent of material was recovered

from the aqueous extracts on the addition of ethanol. Analytical studies showed that these bleached materials were made up almost entirely of polysaccharides based chiefly on glucose and uronic acid residues. The presence of polygalacturonic acids was shown by simultaneous oxidation and hydrolysis to mucic acid.

4. A portion of the pectin was left as part of the insoluble residue when the water-insoluble bleached material was extracted with small volumes of dilute alkali. The presence of calcium in this residue indicated that the pectin was present in the bark as calcium pectate. After decreasing the calcium content by washing the residue with dilute acid, the pectic acid was extracted by dispersion in water. Pure pectic acid having a specific rotation of $+265^{\circ}$ in dilute ammonium hydroxide solution was obtained after several reprecipitations of this material, first as the calcium, then as the ammonium salt.

5. Two different acetates were recovered from the original bleached fraction after acetylation by the pyridine-formamide method. The first of these was insoluble in water, contained only glucose units, and had a specific rotation of $+178^{\circ}$ in chloroform, showing it to be a starch triacetate. This material accounted for zero point fifty-five per cent of the original bark. A second acetate, which formed stable dispersions in water, was identified as a pectin acetate by its uronic acid content. After deacetylation and purification, a

pectic acid was obtained having a specific rotation of +226°. A total of one point fifty-six per cent of the original bark was isolated as pure pectic acid in these two fractions.

6. The starch acetate was also deacetylated with alkali to yield a starch of specific rotation +136° in dilute sodium hydroxide solution. Its identity was confirmed by the characteristic blue colouration with iodine, by acid hydrolysis to glucose, and by enzymatic hydrolysis, using an alpha amylase enzyme, to a mixture of glucose, maltose and maltotriose.

7. When Fraction C was bleached with sodium chlorite, a crude, water-insoluble polysaccharide was isolated in 66 per cent yield. The acid hydrolysate showed this material to be based on glucose, xylose, arabinose and uronic acids, and this mixture was therefore similar to that encountered by Sanderson in the aqueous extract obtained following the liquid ammonia extraction. No attempt was made to resolve the bleached material into pure components, but an enzymatic hydrolysis indicated that at least part of the glucose was present as starch.

8. A further weight loss of 17.0 per cent was obtained when the residue from the large-scale extraction, described in paragraph (2), was exhaustively re-extracted with alkali, and the total amount extracted by alkali was thereby increased to 50.6 per cent of the water-extracted bark, or to 23.8 per cent of the original bark. The material isolated from the extracts amounted to 22.4 per cent of the original. Since a

further weight loss was incurred when the exhaustively-extracted bark was treated with sodium chlorite, delignification was still incomplete. The bleached material was shown to yield mainly glucose on acid hydrolysis, both by the isolation of glucosotriazole, and by paper partition chromatography.

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