URONIC ESTER GROUPS IN WOOD AND STUDIES ON THE "LIGNIN-CARBOHYDRATE COMPLEX"

by

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PART I

SCOPE OF THE PROBLEM

During the past 14 years, the structures of major hemicelluloses in a great number of different wood species have been investigated. Useful generalization can now be made with respect to the amount, nature, and location of the hemicelluloses in our more important trees. It is still evident, however, that a vast number of problems, both large and small, remain to be solved, especially structural details such as the degree of branching of xylans and glucomannans, the distribution of their various side chains, and the state of the uronic acid groups in the native xylans.

The esterification of uronic acid groups in xylan with other wood components has often been speculated upon, but the only ester groups so far established in wood are the acetyl groups in the major hemicellulose components (1). If the uronic acid groups are in fact esterified in their native state in wood, it is important to know whether they are in the form of lactones, cross-links with xylan itself, with other hemicelluloses or with lignin. The last possibility involves the controversial problem of the existence of a linkage between lignin and carbohydrates in wood.

The work described in this thesis had a threefold purpose: (1) to seek direct evidence of the hitherto presumed existence of uronic ester groups in wood; (2) if such groups were proven to be present, to determine whether they constituted cross-links; and (3) to explore the nature of other possible covalent bonds between lignin and carbohydrate by electrophoresis of cleavage products of "lignin-carbohydrate complexes" on glass-fibre paper.

PROBABLE EXISTENCE OF URONIC ESTER GROUPS IN WOOD

One early piece of evidence to support the view that the uronic carboxyl groups were esterified with other wood constituents was obtained by Sarkar and co-workers (2) in an attempt to account for certain properties of jute. They found that treatment with 0.25% sodium hydroxide nearly doubled the acid value of jute as measured by the silver-ion exchange method, and concluded that the additional free acid might have resulted from the hydrolysis of an ester linkage between lignin and polyuronides. Very similar results were reported by Bhattacharjee and Callow (3) who observed that treatment with 0.25% sodium hydroxide increased the acid value of de-ashed jute fibre from 11.4 to 22.5 milliequivalent per 100 g. (determined by silver-ion exchange), and this additional increase was ascribed to the hydrolysis of a uronic ester, a suggestion further supported by the apparently first-order rate of formation of free carboxyl groups. Tachi and Yamamori (4) also found that the carboxyl contents of beech and elm holocelluloses were higher than those of the original woods, and considered that this finding was best explained by the hydrolysis of esterified uronic carboxyl groups.

Foster and co-workers (5) compared the rates of liberation of uronic acid from Eucalyptus regnans wood and its holocellulose, and found that the former reacted much more slowly. Normal sulphuric acid at 91° hydrolyzed 1.45% of uronic acid anhydride in the wood in 3 hours compared to 2.35% in the holocellulose in 1 hour. The authors considered the most likely explanation to be the existence of an ester linkage between the carboxyl groups of uronic acid and a hydroxyl group belonging to other wood components.

The esterification of uronic carboxyl groups in wood has also been considered by other workers who noted that non-extractable nitrogenous material remained in wood meals treated with monoethanolamine or ammonia. Atchison (6) found that the holocellulose prepared by the van Beckum and Ritter method, which claimed a theoretical balance of ash, extractive, lignin and holocellulose, contained a considerable amount of nitrogen; this indicated retention of monoethanolamine. Thomas (7) showed that the retention of nitrogen was as much as 0.3%. Wise and co-workers (8) reported that when holocellulose very low in nitrogen was prepared by the chlorite method, and subsequently treated with an alcoholic solution of monoethanolamine, it showed a marked increase in nitrogen content, thus confirming the general findings of Atchison and Thomas.

Reynolds (9) observed that this retention of nitrogen in the holocellulose was not adventitious. Lignin-free holocelluloses prepared from spruce, American white oak and Eucalyptus regnans by chlorination and extraction of the chlorinated lignin with monoethanolamine or alcoholic ammonia, contained 0.3 to 0.5% of nitrogen which could be removed by hot 0.04N sodium hydroxide. Reynolds suggested that the action of chlorine, which was shown to be a necessary stage in the formation of the nitrogen compound, cleaved a labile glycosidic linkage and, at the same time, oxidized the newly-generated reducing end-group to a lactone. The latter then reacted with alcoholic monoethanolamine or ammonia to give an acid amide.

Yan (10) found similar retention of nitrogenous material in residue from sugar maple wood meal treated with liquid ammonia. Later

Neubauer (11) extracted this residue exhaustively with ethanol and then with hot water. The extraction removed 0.8% of a lignin and 1.9% of a crude hemicellulose, but did not remove the nitrogenous material. Neubauer showed that when samples were distilled with dilute aqueous sodium hydroxide, the surplus nitrogen was removed as ammonia, and, therefore, had been present in the wood residue as an amide or an ammonium salt. The latter possibility was considered improbable because boiling aqueous magnesium hydroxide expelled no more ammonia from the residue than did boiling water. Moreover, acetamide could not have been the source of the residual nitrogen, since it would have been removed by the exhaustive extraction. Rather, Neubauer suggested that some of the uronic acid groups in the original wood had possibly been trans-esterified with other constituents; cleavage of these ester groups by liquid ammonia had made it possible to extract small additional quantities of lignin and carbohydrate material by the renewed use of alcohol and hot water, while the insoluble amides remained in the residue.

Timell (12) extracted a white birch chlorine holocellulose with dimethyl sulphoxide and isolated, in a yield of 50%, a salt-free, water-soluble O-acetyl-4-O-methylglucurono-xyloglycan containing one α-(1-2)-linked 4-O-methyl-D-glucuronic acid residue and 3.6 acetyl groups per 10 xylose residues. From periodate oxidation, the infrared spectrum, sodium borohydride reduction and viscosity behavior experiments, he concluded that the uronic acid groups were neither lactonized nor ionized, but he did not rule out the possibility that these groups might be esterified with hydroxyl groups on the main chain of the xylan.

In a few cases, partial hydrolysis of aspen (13) and yellow

birch (14) woods have yielded glucofuranosylurono-(6-3)-lactone. However, this could not be regarded as evidence supporting the theory of lactone formation in uronic acid, because Roudier and Gillet (15) had demonstrated that 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose was slightly demethylated under conditions normally used for the hydrolysis of polysaccharides. The small amounts of glucurono-lactone detected in hydrolyzates from both hardwoods could therefore well be artifacts.

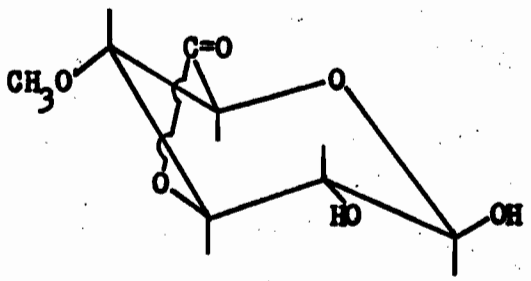
Although much indirect evidence strongly suggests the existence of glucuronic ester groups in wood, it cannot be regarded as conclusive. Direct evidence is therefore required to show their actual presence.

ORIGIN OF THE HYDROXYL GROUP ESTERIFIED WITH 4-O-METHYLGLUCURONIC ACID

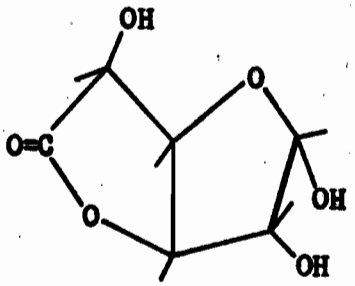
If, in fact, uronic carboxyl groups are esterified in their native state, there are four possible modes of combination: The first is simple lactonization involving the C-3 hydroxyl group of the same monomer unit on which the carboxyl group occurs. Opposed to this idea is the fact that the existence of a 4-O-methylglucopyranosylurono-(6-3)-lactone (I) has never been reported in the literature. Attempts to prepare this lactone by vacuum distillation of the acid ended in failure (16). Steric factors (17) favour the formation of glucofuranosylurono-(6-3)-lactone (II) which can be easily prepared from glucuronic acid.

The etherification of the C-4 hydroxyl group in the glucuronic acid side chain of xylan prevents the transformation into the furanose form, and therefore, minimizes the possibility of lactonization.

The second possible mode of combination is the intramolecular



(I) 4-O-Methylglucopyranosylurono-(6-3)-lactone (not known)



(II) Glucofuranosylurono-(6-3)-lactone (easily prepared)

Figure 1 Glucuronolactones

esterification of uronic acid with xylose hydroxyl groups from the same xylan molecule.

The third possibility is that the uronic ester forms cross-links with hydroxyl groups of lignin. Many workers (2, 3, 5), who have reported indirect evidence supporting the presence of glucuronic ester groups in wood have favoured the postulation of such a cross-link. The chemical properties and morphology of the wood also do not exclude such a possibility. However, the actual presence of intermolecular esterification between uronic acid and hydroxyl groups of lignin has not yet been rigorously demonstrated.

The fourth possibility is intermolecular esterification with hydroxyl groups belonging to other carbohydrate constituents of the wood. From chemical point of view, this appears to be the most probable alternative, since a large number of free hydroxyl groups is available for esterification.

PROBABLE CROSS-LINKS BETWEEN WOOD CONSTITUENTS

The presence of cross-links between major components of wood has been speculated upon for some years. Many (18) consider the understanding of such cross-links is essential to wood chemists seeking methods to separate cell wall components in nearly quantitative yield, whether the purpose of separation is analytical or commercial. While others (19) believe the positive identification of such cross-links will help to explain many puzzling properties of the wood. Schuerch (20), however, regards the actual presence of cross-links in wood to be of minor importance, because substantial gains in our knowledge of cellulose,

hemicellulose and lignin have been made without considering the presence of lignin-carbohydrate bonds.

The controversy and confusion of the problem may be attributed to many irresponsible statements in the literature published during the past hundred years since the days of Payen's "incrustation" theory and Erdmann's "glycolignose" theory. Many wood chemists are anxious to claim the credit for providing evidence and a solution. Oscillation of opinion between either theory during the careers of many prominent wood chemists has not been uncommon. While others, due to poor understanding of the three major components of wood, have only created further complications.

Exhaustive surveys of the literature related to this problem have appeared in many excellent reviews (18, 21, 22), but none has been critical, especially of the experimental technique employed by the numerous workers. A comprehensive review of pertinent literature on the lignin-carbohydrate linkage will not be repeated here, but a critical evaluation of the various experimental techniques employed in the study of this problem will be briefly treated.

(1) EXTRACTION AND SEPARATION OF CELL WALL COMPONENTS

The most important original justification for the concept of a lignin-carbohydrate bond is that hemicellulose, cellulose and lignin or their derivatives are much less soluble when they are in the wood than after isolation. Recent work of Nelson and Schuerch (23) has shown that mutual physical restraint between polysaccharides and lignin is sufficient to explain the observed differences in solubility between native and isolated polysaccharide, and the postulation of a lignin-carbohydrate bond is not actually necessary. Nelson and Schuerch also pointed out

the important fact that the extractability of hemicellulose, particularly xylan, varies very greatly among different species of wood, even though their structures are similar. Therefore, evidence suggesting the presence of a lignin-carbohydrate bond based on the differences in solubility cannot be taken seriously.

Covalent bonds of the ether type between hemicellulose and cellulose have also been postulated primarily to explain the presence of extraneous sugars in purified cellulose. However, the shortcomings of fractional precipitation methods have been demonstrated by Snyder and Timell (24) in their study on balsam fir cellulose, and extraction methods have now been improved to the point where wood celluloses containing a fraction of a percent of extraneous sugars can be prepared (25, 26). The question of covalent bonds between extraneous sugars and the cellulose of wood, therefore, has become more remote.

Typical confusions in the study of this controversial problem can be found in the acetolysis and chlorination of wood. Evidence suggesting a combination between xylan and lignin in beech wood was claimed by Kawamura and Higuchi (27). Beech wood meal was treated for 24 hours at room temperature with an acetic acid-acetic anhydride mixture (2:1) containing a trace of sulphuric acid. "Acetyl xylo-lignin" containing 27.2% Klason lignin and 15.8% pentosan was then extracted with chloroform and acetone in 40% yield. The "xylo-lignin" was de-acetylated with 0.1 N sodium hydroxide and resolved by ascending paper chromatography with a n-butanol:acetic acid:water (4:1:1) system. The lignin spots with R_f = 1.0 were cut and eluted with acetone. Hydrolysis of the lignin followed by paper chromatography indicated the presence of xylose, and methylation followed by hydrolysis and paper chromatography yielded

2,3-di-O-methyl-D-xylose and 2,3,4-tri-O-methyl-D-xylose. Kawamura and Higuchi considered the presence of trimethyl sugar in the hydrolyzate provided convincing evidence for the existence of a phenolic glycosidic linkage in wood. However, the trimethyl xylose could well be derived from entrained xylobiose. When critically evaluating these results, the validity of several experimental procedures must not be overlooked. Hayashi and Tachi (28) showed that an "acetyl xylo-lignin" isolated from wheat straw by the same method could not be completely de-esterified with 0.1 N sodium hydroxide. The de-acetylated "xylo-lignin" of Kawamura and Higuchi may, therefore, have been a partially acetylated "xylo-lignin". The chromatographic mobility of acetyl polyxylose was not mentioned by the authors. The effectiveness of the solvent system used to separate partially acetylated lignin and polyxylose was not demonstrated. Possible condensation between xylan and lignin by "reversion" (29) during extraction of the "acetyl xylo-lignin" was also not considered. Although Kawamura and Higuchi have presented some evidence supporting the presence of a combination between xylan and lignin, it is dubious that they had actually achieved a complete separation of a physical mixture containing carbohydrate and lignin.

Widely acclaimed evidence in favour of a lignin-carbohydrate complex in wood was also reported by Traynard and co-workers (30) from studies on the chlorination of poplar wood under anhydrous conditions (anhydrous solvent not given). Three fractions were obtained from the chlorinated wood by successive extractions with (1) cold ethanol (2) hot ethanol (3) cold alkali (concentration not given). The fractions extracted by cold ethanol and cold alkali were acetylated and then "purified" by chromatographic adsorption on a column of alumina. The

three chlorolignins extracted previously from the wood and the two "purified" acetate fractions were then hydrolyzed with 1 N hydrochloric acid. Parallel paper chromatograms showed that in each case, sugars were absent from the unhydrolyzed sample and were present after hydrolysis. The sugars found consisted mainly of xylose, with smaller amounts of arabinose and galactose. These results appeared very interesting and had been mentioned in many reviews (18) as convincing evidence supporting the existence of a union between lignin and xylan in wood. Upon critical evaluation, many defects are apparent. The experimental procedure, yields of the chlorolignin extracted and the pentosan contents were not given in the original publication. The solvent system used in paper chromatography was not clearly stated and the resolution of the chromatographic methods were not unequivocally demonstrated. Therefore, like the evidence of Kawamura and Higuchi, the results presented by Traynard and co-workers, which assumed the complete physical separation of carbohydrate and chlorolignins in their unhydrolyzed samples must be viewed with caution.

(2) MICROBIOLOGICAL OR ENZYMIC DEGRADATION OF WOOD CONSTITUENTS

Isolated polysaccharide materials have been reported (18) to be easily and completely degraded by micro-organisms or enzymes while polysaccharide in wood is only degraded to a limited extent. This observation has often been considered as evidence for a lignin-carbohydrate bond in wood. However, Painter (31) has recently shown that in pure xylan there is certain fraction (4%) of the polymer which is resistant to the action of enzyme, although most of the xylan is hydrolyzed under such condition in a very short time (less than 24 hours). Jones and co-workers (32) have reported that a xylan containing 2.9% of lignin was completely resistant to degradation by the enzyme hemicellulase.

The resistance of certain portion of polysaccharide in wood towards the action of enzymes can be interpreted in many ways without postulating a lignin-carbohydrate bond. From the basic kinetic point of view, it can be considered that the portion of "reactive sites" on the polysaccharide substrate is increasingly diminishing as the degradation proceeds in the presence of a non-hydrolyzable material, lignin. As a result, the chance of collision between these "sites" and the enzyme become much less than at the initial stage of the reaction. The chance of collision (33), expressed in terms of collision number, Z, is a complicated function of the average distance between the centres of collision, concentrations and molecular masses of the substrate and the enzyme. The presence of highly polymerized lignin might further interfere with the specificity of the enzyme on the remaining "reactive sites" of the polysaccharide. In light of the complex nature of the enzyme-catalyzed heterogeneous degradation of polysaccharide, it is hard to conceive that the resistance of polysaccharides in wood towards enzyme action could be singled out as resulting from the presence of lignin-carbohydrate bonds. Further, the resistance of such a bond towards enzyme action has never been demonstrated. Therefore, results suggesting the existence of a lignin-carbohydrate bond based on enzymic degradation experiments provide only a retreat from the problem itself and not a solution; they must be interpreted with caution.

PART II

AMMONOLYSIS OF URONIC ESTER GROUPS IN WHITE BIRCH XYLAN AND OTHER WOODS

GENERAL INTRODUCTION

Ammonolysis in liquid ammonia was the method chosen to detect the presence of ester links. Excellent reviews on the properties and reactions of liquid ammonia have already been published (34, 35), so only effects of interest to this thesis will be briefly mentioned here. In general, liquid ammonia resembles alcohol in its solvent powers towards organic substances. Most aliphatic and aromatic ethers, aldehydes and ketones are soluble in liquid ammonia, but the carbonyl compounds frequently react to give imino derivatives (34). Carboxylic acids, such as acetic and benzoic acids, are dissolved as ammonium salts, but higher aliphatic acids and dicarboxylic acids are rather insoluble. However, treatment of an acid, such as acetic, or an acid anhydride, with liquid ammonia at higher temperature and pressure results in the formation of acid amide (36). Most acid amides and esters are quite soluble, but under pressure at room temperature, esters undergo ammonolysis to give the corresponding amide.

REACTIONS OF SIMPLE CARBOHYDRATES WITH AMMONIA

Glycosides of the ordinary sugar as well as their acetylated and isopropylidene derivatives are quite soluble in liquid ammonia at its boiling point (37). No reaction occurs at this temperature, but at 25° under pressure, de-acetylation takes place with the formation of "aldose amide" (38). The reducing sugars, when dissolved in liquid ammonia at -33°, form glycosylamines through an aldehyde addition product (37). Liquid

ammonia has also been used as a solvent in the preparation of sodium-alcoholates from polysaccharides for micromethylation using methyl iodide (39)

REACTIONS OF CELLULOSE WITH AMMONIA

Anhydrous liquid ammonia at its boiling temperature (-33°) is, like water, neutral in reaction, and possesses high solvent powers with an ability to swell cellulose. The physical effect of liquid ammonia on cellulose has been the subject of many extensive reviews (40, 41).

Cellulose swollen by liquid ammonia possesses different crystalline modifications, as shown by the x-ray diffraction patterns (42). Ramie fibres gives two ammonia-celluloses, mutually interconvertible between -20° and -30°. One form of the ammonia-cellulose (ammonia-cellulose II) is stable at lower temperatures; when it is warmed, it changes rapidly to ammonia-cellulose I, stable above -20°, but the reverse change is slow. Loss of ammonia from ammonia-cellulose gives a third modification (ammonia-cellulose III) which closely resembles the cellulose mercerized by caustic soda. This ammonia-cellulose III can be converted more easily and completely to neutral cellulose by boiling with dilute acid or alkali at ordinary pressure. The results are summarized in Figure 2.

The retention of ammonia by cellulose after such treatment has been shown recently (43) to be the result of hydrogen bond formation between the hydroxyl groups of the cellulose and the nitrogen of the ammonia (44). The treated samples show increased reactivity to acetylation, hydrolysis and ethanolysis and reduced resistance to thermal treatment. A comparison with mercerized cellulose indicates that the effect of liquid ammonia in increasing the reactivity is considerably greater than the effect of sodium hydroxide. However, Sanyer and Purves

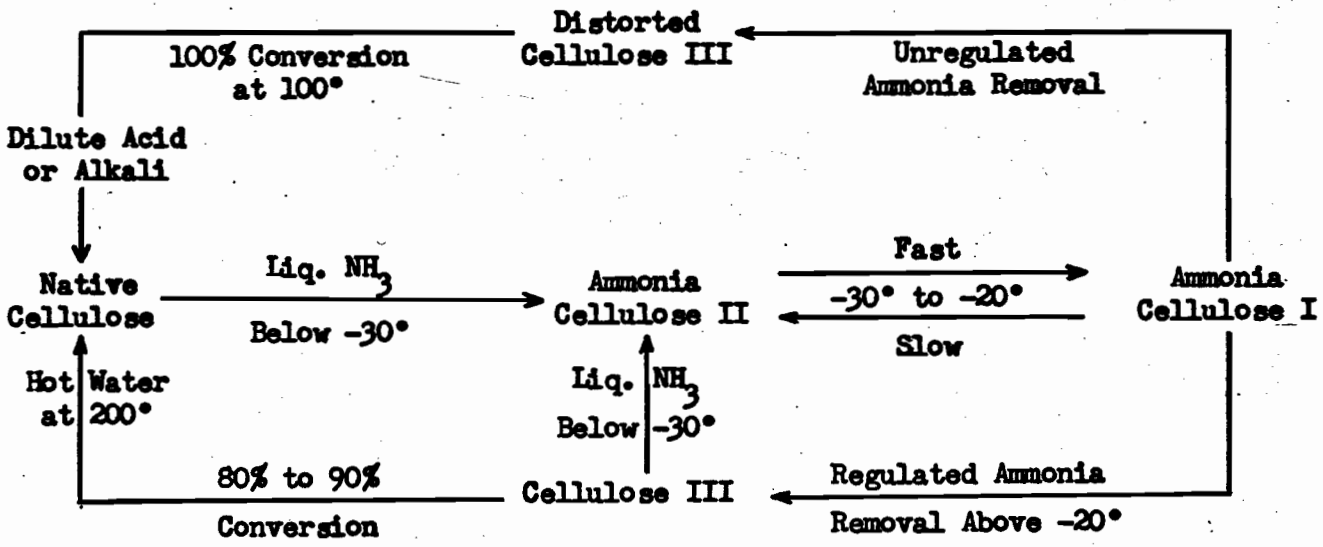


Figure 2 Crystal Forms of the Ammonia Celluloses (42)

(45) have shown that liquid ammonia could degrade a cotton cellulose from a $D.P._n$ of 3,000 to 2,888 under similar conditions.

The ability of liquid ammonia to swell native cellulose in wood was demonstrated by Schuerch (46) who plasticized a birch veneer strip (tongue depressor) by immersion in liquid ammonia to the extent that it could be twisted into a helix by finger pressure without cracking. Upon evaporation of ammonia, the wood strip retained the imposed shape with little change in mechanical properties, but with some lateral and longitudinal shrinkage and some changes in moisture-regain characteristics. Schuerch suggested that treatment with liquid ammonia probably broke the hydrogen bonds within both the amorphous and crystalline regions of the polysaccharide cell wall and the lignin matrix; the evaporation of the ammonia re-formed the hydrogen bonds in the new position and resulted in a permanent set.

The swelling effect of liquid ammonia can also be used to improve the extensibility of paper. Arlov and Snaprud (47) showed that rapid evaporation of ammonia from the treated paper caused the sheet to shrink in all directions of the plane. The modified paper also showed marked improvements in other physical properties such as tearing strength, bursting strength and folding endurance.

REACTIONS OF WOOD WITH AMMONIA

Consideration of the mild chemical action and the versatile solvent power of anhydrous liquid ammonia, together with its great ability to swell and penetrate the crystalline and amorphous portions of cellulose, led Purves and his students (40, 41) to study the action of liquid ammonia on wood.

Early work disclosed that liquid ammonia under pressure (~150 psi) at room temperature removed small amounts of lignin and polysaccharide from maple wood meal (10). From the bark of white spruce, ammonia extracted small fractions of carbohydrate materials, fatty acid amides, and complex phenolic compounds (48). A substantial amount of acetamide was also recovered from the liquid-ammonia extract as a result of ammonolysis of the carbohydrate acetates in the original wood or bark. In addition, a small portion of the residue from the extraction with liquid ammonia was rendered soluble in hot water (11). This solubilization was tentatively attributed to the cleavage of ester groups of an unknown type by liquid ammonia, because the residual wood showed an increase in nitrogen as amides (40, 41).

The pioneering work on liquid-ammonia extraction carried out at McGill University drew much interest and attention from wood chemists in other laboratories, and they began to use this reagent either as a mild solvent or as a pre-treatment to increase the yield of later extractions of hemicellulose by other solvents. Bjorkqvist and Jorgensen (49) found that treatment of spruce holocellulose with liquid ammonia made some previously water-insoluble polysaccharide material extractable by hot water. The hot-water soluble hemicellulose contained 45% of mannose and lesser amounts of glucose, xylose, galactose, arabinose and uronic acid units. Extraction of the residue with dilute alkali removed more of the hemicellulose but left about 10% of mannose in the pulp. Bishop and Adams (50) soaked a wheat straw holocellulose in liquid ammonia for 36 hours at -33°. Subsequent fractionation by successive extractions with cold water, 0.5% sodium carbonate, 0.5% and 2.2% potassium hydroxide showed that pretreatment with liquid ammonia increas-

ed the yield of material soluble in cold water from 3% to 20.2%. Analyses of the isolated fractions showed some systematic differences, with pentosan contents increasing and uronic acid anhydride decreasing progressively in the alkali soluble fractions. In a later publication (51) Bishop showed that 8.0% of the wheat straw holocellulose can be extracted by anhydrous liquid ammonia alone at -33°. The extract consisted of (a) acetamide (b) a polyuronide fraction, 1.3% of the holocellulose, containing D-xylose, L-arabinose, D-glucose, D-galactose, and D-glucuronic acid in approximate molar ratios of 11: 3: 3: 1: 2.5, and (c) a non-carbohydrate portion consisting of low molecular weight degradation products among which methylamine was identified after hydrogenation. Bishop also showed that anhydrous liquid ammonia degraded an isolated polyuronide by 8.4%.

Marchessault, Glaudemans and Cafferty (52) made use of the physical effect of anhydrous liquid ammonia on holocellulose by employing shorter time (30 seconds) of treatment. A birch chlorite holocellulose treated this way was extracted with hot water and gave a xylan which retained most of its original acetyl groups. The yield was found to be much higher than that from a direct extraction of the holocellulose with dimethyl sulphoxide. Hot water extraction of a liquid-ammonia-treated pine holocellulose gave a mixture of arabino-glucuronoxylan and acetylglucomannan.

So far, the additional nitrogen, analyzed as amides, found in the wood after liquid ammonia extraction does not seem to have received much attention. Yet, it is clear that successful isolation of this material containing amide nitrogen can throw much light on the native state of the glucuronic acid groups as they exist originally in the wood.

HISTORICAL INTRODUCTION

Prior to the series of fundamental studies on the extraction of wood, bark, and holocellulose with anhydrous liquid ammonia initiated in 1944 in this laboratory, a limited amount of similar work had been carried out in other countries. Peterson and Hixon (53) at the Iowa State college studied the pulping of cereal straw by aqueous ammonia at pressures not exceeding 100 pounds per square inch which closely resembled the conventional aqueous alkali pulping process. In Germany, Freudenberg and co-workers (54) investigated the action of alkali metals and their amides dissolved in liquid ammonia on a limited variety of lignins and woods. After such treatment at room temperature, lignin fractions became soluble in alkali and absolute methanol. From reactions of model compounds, it was shown that phenolic ethers underwent partial or complete demethylation to yield phenols. Shorygina and co-workers (55) have demonstrated recently that aqueous ammonia (24%) at 180° can also cause demethylation of chlorolignins and at the same time replace all the chlorine with amino groups.

The early experiments of Freudenberg and his colleagues led Yan (40) to investigate the use of commercial anhydrous liquid ammonia alone as a "mild" solvent for lignin. Extractions with liquid ammonia at 25° and about 150 psi pressure removed more material from hardwood than from softwood. Yan, therefore, concentrated on studying the action of liquid ammonia on maple wood (Acer saccharum, M.). Liquid ammonia extracted 5 to 7% by weight of the wood, the extract being separated into three fractions differing in solubilities in methanol. The first fraction insoluble in methanol, 0.8%, consisted of polysaccharide material. The second methanol-soluble fraction was shown to be acetamide, in an

amount almost equivalent to the 3.4% of acetyl groups removed from the original wood. The remaining fraction, "liquid-ammonia lignin," was recovered in 0.7% yield after removal of the acetamide by chloroform, and represented 3% of the Klason lignin in wood. Yan found that the nitrogen content of the "liquid-ammonia lignin" was negligible. However, Enkvist and co-workers (56) have recently shown that it was possible to introduce 3 to 12% of nitrogen into spruce sawdust and sulphate lignin by treatment with liquid ammonia at 230°.

The nearly quantitative production of acetamide from the acetyl froups in the wood showed that ester links were cleaved by liquid ammonia. Cleavage of any other kind of ester links, possibly between hydroxyl groups of lignin and uronic carboxyl groups, would result in chemical changes in the residual wood and might lead to solubilization of previously insoluble components. In such a process, any amide groups formed would be found with the carbohydrate portion of the wood. Analysis of the residual wood, indeed, showed an increase of 0.33% nitrogen.

Neubauer (11), therefore, investigated the possibility of extracting previously insoluble material from liquid-ammonia-extracted maple wood prepared by Yan. Extraction of the wood with hot water removed about 2% of the residue. The extract contained a polysaccharide with D-xylose units and methoxylglucuronic acid highly contaminated with pectin material. Yet, the residue, even after exhaustive extraction, retained the 0.33% nitrogen originally present in the liquid-ammonia-extracted wood; analysis showed that the amide nitrogen content of this wood was 0.25%.

By means of hot water, similar hemicellulose fractions were

also extracted by Milks (57) and Milford (58) from aspen or black spruce holocelluloses previously treated with liquid ammonia. These results indicated that hemicelluloses could be extracted under milder conditions than those employed conventionally using strong alkali. In Jablonski's study of the action of liquid ammonia on extractive-free white spruce bark (48), about 3% was extracted in 24 hours by liquid ammonia at room temperature under pressure and the extract could be fractionated into n-butanol-soluble and insoluble portions. The butanol-soluble fraction (1.76%) contained acetamide, in an amount corresponding nearly quantitatively to the acetyl content of the original bark, and a complicated mixture of long chain acid amides, among which only lignoceric acid amide was identified. The butanol-insoluble fraction (1.06%) contained a mixture of polysaccharides; after acid hydrolysis, paper chromatography identified the presence of galactose, glucose, mannose, arabinose, xylose, and uronic acids.

With these studies in the background, the present research began with an evaluation of the conditions which give the maximum amide nitrogen content in the wood after treatment with liquid ammonia.

RESULTS AND DISCUSSION

OPTIMAL DURATION OF AMMONOLYSIS OF WHITE BIRCH WOOD MEALS

In order to evaluate the optimal duration of ammonolysis to yield the highest amide nitrogen content of the treated wood, small samples of extractive-free birch wood meal were treated with excess anhydrous liquid ammonia at room temperature under pressure (~150 psi) for periods up to 100 hours. The yields of the liquid ammonia extract of a sample treated for 100 hours (4.6%) was only slightly higher than that after 1 hour (3.9%).

At the end of each period, the reaction was stopped by cooling to -65°, and the ammonia was removed. The residue was thoroughly extracted with methanol to remove acetamide derived from ammonolysis of the acetyl groups present in the native xylan. As in the previous work, the quantitative differentiation of amide from ammonium nitrogen was based on the selective expulsion of ammonia from the latter by boiling with an aqueous suspension of magnesium oxide (59) which had a limited solubility of 0.0086 g. in hot water and a pH of 10; a conventional titration of the ammonia in the distillate determined the ammonium nitrogen. Modified equipment made it possible to determine amide nitrogen in the same way, and on the same sample, by a subsequent distillation with 60% aqueous sodium hydroxide. Control experiments with known mixtures of pure acetamide and ammonium acetate showed the method to be reliable. The Kjeldahl nitrogen content of the untreated wood was 0.06%, and of this, 0.04% which responded as amide could probably be ascribed to nitrogen in the wood protein (60).

Table 1 shows that the amide nitrogen contents of the wood in-

TABLE 1

Content of Amide and Ammonium Nitrogen in Samples Treated near 20° with Liquid Ammonia under Pressure (about 150 p.s.i.)

Sample	% Nitrogen[a] Ammonium	Amide	% Soluble[b]
A. Birch wood meal			
(i) before ammonolysis[c]	0.01	0.03	-
(ii) after ammonolysis for			
1 hour	0.08	0.13	3.9
2 hours	0.14	0.16	-
5 hours	0.10	0.18	-
10 hours	0.14	0.22	-
15 hours	0.09	0.23	-
25 hours	0.10	0.25	-
50 hours	0.12	0.26	-
100 hours	0.12	0.25	4.6
B. "Xylan-poor" wood meal[d] after ammonolysis for 72 hours	3.45	0.03	-
C. Extracted birch xylan[e] after ammonolysis for 72 hours	0.23	0.01	-
D. Mixture (3:1) of (B) and (C) after ammonolysis for 72 hours	2.03	0.03	-

(a) Calculated on oven-dry wood.

(b) Soluble in liquid ammonia.

(c) After extraction of original meal with 24% potassium hydroxide under nitrogen.

(e) Recovered from the potassium hydroxide extract.

creased gradually with time of ammonolysis until a maximum value of 0.25% was reached after 25 hours. In view of the maximum amide nitrogen content, the ammonolysis time of 5 hours evaluated as optimal with respect to the yield of liquid ammonia extract by Yan (40) was not adopted in the present experiment, and instead, a period of 72 hours was chosen in subsequent experiments.

RELATIONSHIP BETWEEN THE URONIC ACID AND NITROGEN CONTENTS AFTER AMMONOLYSIS

Table 1 showed that ammonia treatment caused the ammonium nitrogen content of the wood to attain its final constant value of 0.11 ± 0.03% within an hour. If this value corresponded to the uronic acid present in the extractive-free birch wood as a free acid or inorganic salt, it gave an ammonium salt to amide mole ratio (i.e. 0.11: 0.25) of about 3: 7, and the sum of nitrogen, 0.36% was equivalent to 4.5% as uronic acid. The reported uronic acid content of white birch wood determined by the de-carboxylation method (61) was 4.6% (62). Similar agreement was also obtained from liquid-ammonia-treated aspen, balsam fir, esparto grass, jack pine, and tamarack woods as shown in Table 2. This excellent agreement was probably fortuitous, because yield of residue from liquid ammonia extraction was in all cases about 92%, although the amount of lignin and polysaccharide materials removed by liquid ammonia total less than 2% (40). Furthermore, prior extraction of the white birch wood meal with strong (24%) aqueous potassium hydroxide yielded a residual wood B and an extracted xylan C both of which were practically devoid of amide nitrogen, presumably because the alkali had hydrolyzed all ester or lactone groups originally present. Yet the ammonium nitrogen contents (Table 1) of the highly swollen wood residue B (3.45%) and that of the extracted

TABLE 2

Estimation of Uronic Acid from Nitrogen Contents

Wood	% Nitrogen			% Uronic Acid		
	Ammonium	Amide	Total	Calc. from N	Lit. Value	Reference
Birch	0.11	0.25	0.36	4.54	4.60	(62)
Balsam Fir	0.17	0.10	0.27	3.40	3.40	(62)
Aspen	0.14	0.08	0.22	2.77	3.30	(62)
Jack Pine	0.12	0.10	0.22	2.77	3.90	(62)
Tamarack	0.13	0.10	0.23	2.90	2.90	(62)
Esparto Grass	0.08	0.16	0.24	3.02	3.10	(63)

xylan C (0.23%) had no obvious connection with their content of uronic acid. The significance of the values for "ammonium" nitrogen after liquid ammonia treatment requires further study.

CONFIRMATION OF THE PRESENCE OF AMIDE IN AMMONOLYZED WHITE BIRCH WOOD BY INFRARED SPECTROSCOPY

Confirmation of the presence of amides in the ammonolyzed wood, free of acetamide, was obtained by infrared spectroscopy. Bolker and Somerville (64) had earlier applied differential spectroscopy to determine the spectra of lignins in whole woods and pulps; in the present work the technique was altered, and the differential spectra of holocelluloses were determined by using wood in the sample beam and precisely calculated quantities of dioxane lignin (65), from the same wood, in the reference beam of the double-beam spectrometer. As shown in Figure 3, the holocellulose differential spectrum (I) corresponded very well to the spectrum (against air) of isolated Tappi holocellulose (II). In the differential spectrum there are slight, but seemingly unavoidable, shifts of some of the principle bands, and also slight residues of the lignin spectrum, particularly at 1,500 and 1,600 cm^{-1}. However, these defects are not significant when the differential spectrum of the holocellulose in liquid-ammonia-treated wood (III) was observed. Here, as expected, the absorption bands at 1,730 cm^{-1} and 1,250 cm^{-1}, characteristic of acetyl groups, were absent, but a new, very strong band had appeared at 1,680 cm^{-1}. This band corresponded to the amide I band of α-D-glucuronamide (Figure 4, V; IV is the spectrum of glucofuranosylurono-(6-3)-lactone for comparison), although the latter was found at a somewhat lower wavenumber (1,655 cm^{-1}). The absorption of the amide I band in infrared spectra of polymers at a higher frequency than the usually reported wave-

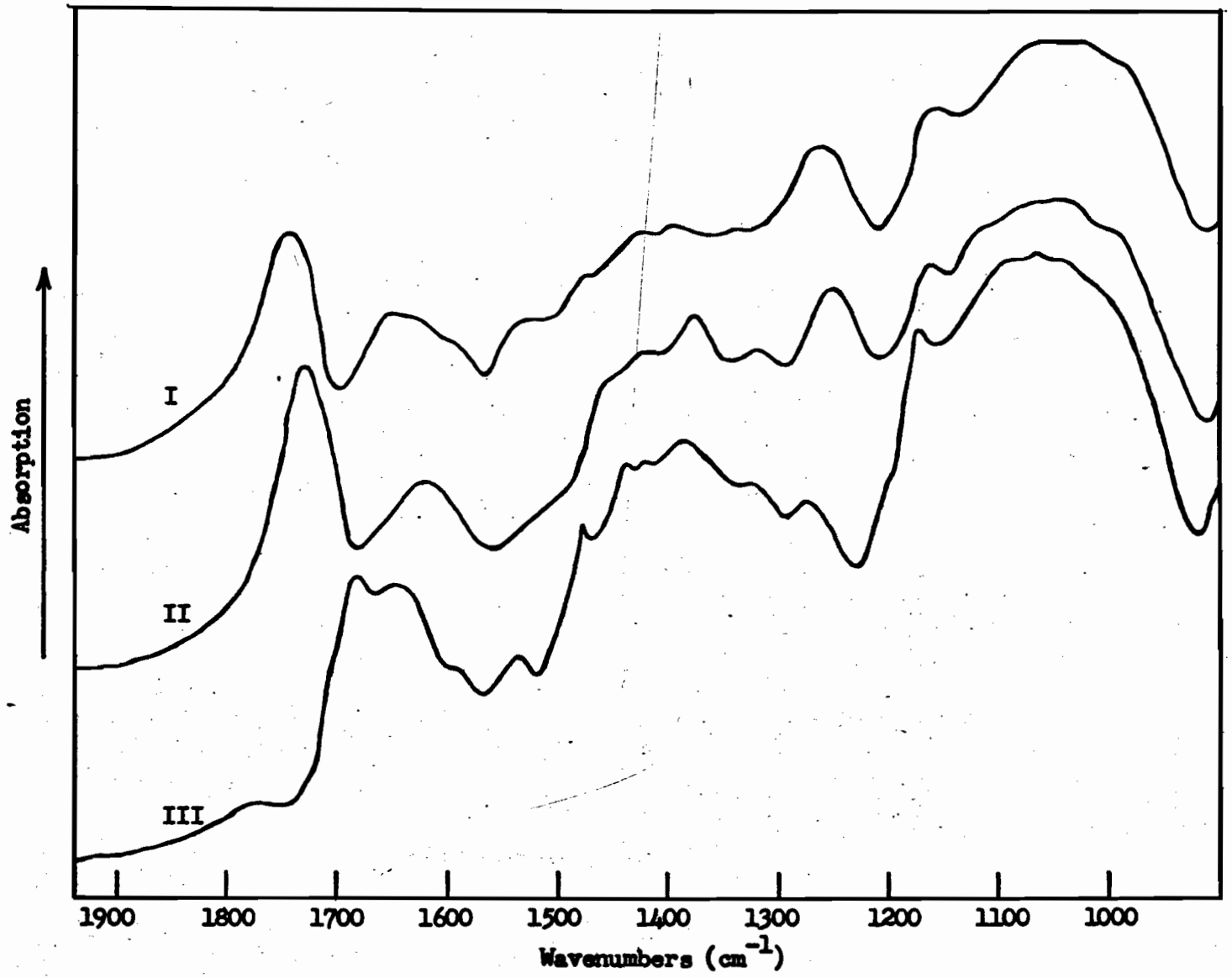


Figure 3 Differential Infrared Spectrum of Holocellulose in Untreated Birch Wood (I), Infrared Spectrum of Birch Holocellulose (II), and Differential Infrared Spectrum of Holocellulose in Birch Wood Treated with Liquid Ammonia (III).

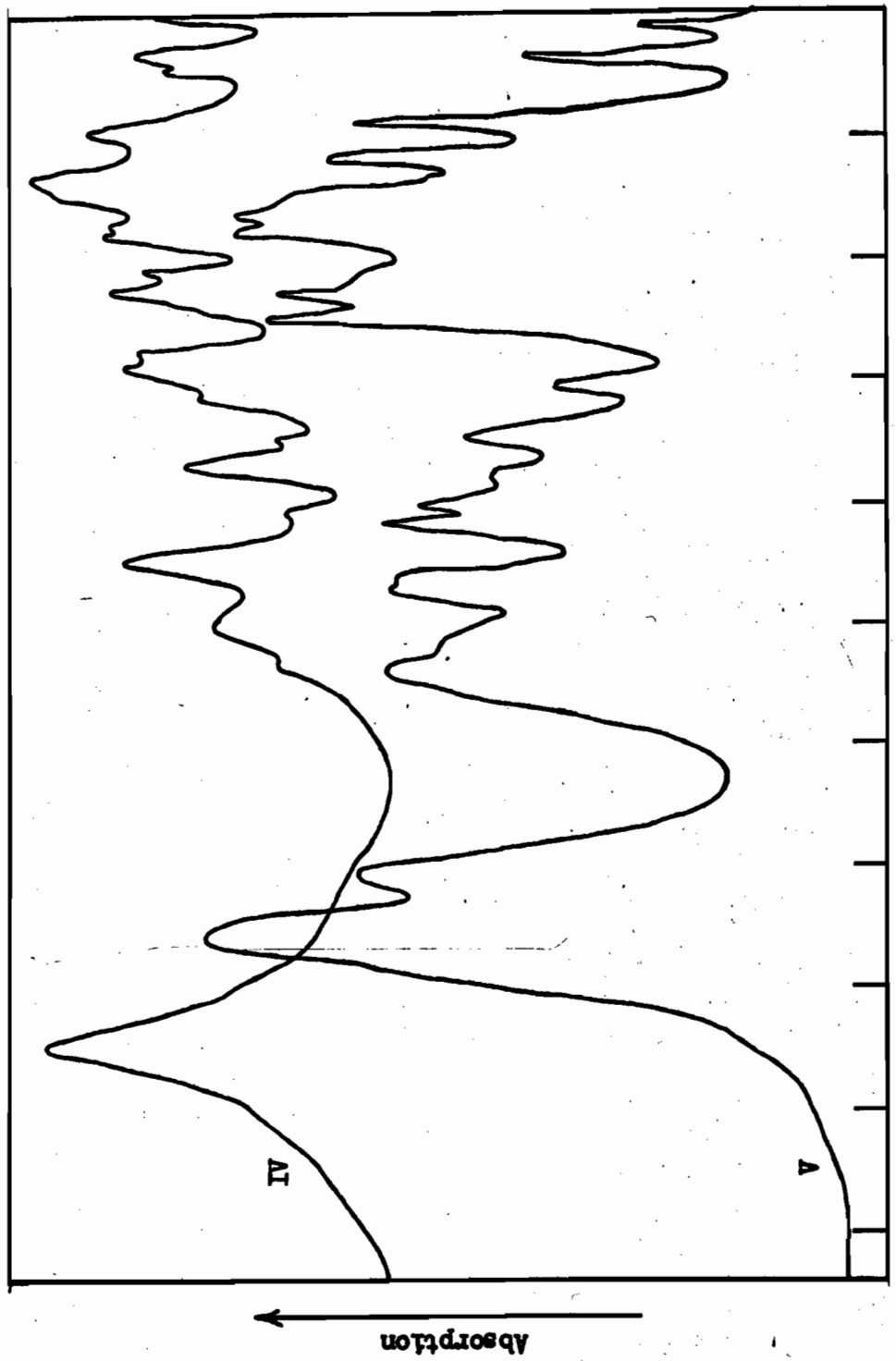


Figure 4 Infrared Spectra of Glucuronolactone (IV) and α-D-Glucuronamide (V)

number (near 1,650 cm^{-1}) (66) for simple primary amides in the solid state was also noted for deoxyribonucleic acid (67) where the corresponding amide I band was observed at 1,677 cm^{-1}. The amide II band at 1,608 cm^{-1} (Figure 4, V) could not be seen in the holocellulose spectrum, probably because of overlapping by the ionized carboxyl band (near 1,610 cm^{-1}) in the same region.

ISOLATION OF "XYLAN AMIDE" FROM LIQUID-AMMONIA-TREATED WHITE BIRCH

After the treatment with liquid ammonia and preliminary spectroscopic analysis, attempts were made to extract the "xylan amide" from the wood. Because of the reactivity of amides towards acid or alkali, the procedure of isolation could employ neutral solvents only. Dimethyl sulphoxide had been reported on several occasions (see Appendix) to be an excellent solvent for extracting hemicellulose, particularly acetylxylan, from holocellulose. Although the extraction was tried for 42 hours under nitrogen, both at room temperature and at 80° with solid: liquor ratio of 1: 15, only about 3.5% of the wood constituents was dissolved (see Appendix).

Attention was then turned to exploring other methods of isolating the "xylan amide" which would preserve the amide groups. Pew (68) had already shown that if a wood sample was ground for 5 hours and then subjected to the action of cellulytic enzymes, about 95% of the carbohydrate material was made soluble in water. Accordingly, Pew's method was then applied to the liquid-ammonia-treated white birch using a 10% aqueous solution of pectinase. At the end of the reaction period (4 days), the solution was transferred into a cellophane dialysis bag and dialyzed against distilled water to separate the hydrolyzate. However, the con-

centrated enzyme solution enclosed in the bag acted on the cellophane membrane and caused it to rupture in 2 hours. Attempts were then made to precipitate the enzyme before dialysis by boiling the solution for 1 hour as recommended by the supplier of the enzyme. However, boiling only resulted in a highly viscous brown solution with no sign of precipitation. Enzymic hydrolysis of the milled liquid-ammonia-treated wood using Painter's method (31) also ended in failure.

It has been reported in the literature (69) that some amides were quite stable in boiling 0.1 N hydrochloric acid. This suggested that chlorination of the liquid-ammonia-treated wood suspended in cold (0°) carbon tetrachloride might facilitate subsequent extraction of the "xylan amide" by neutral solvents. One g. of the wood was chlorinated three times at five minutes each time in excess (50 ml.) carbon tetrachloride. Later larger quantity of carbon tetrachloride was used because it was observed that the organic solvent turned bright yellow in colour after chlorination obviously due to the solution of chloro-lignin. It was considered that in this way, much of the chloro-lignin might be dissolved without using a alcoholic solution of monoethanolamine which was known to introduce nitrogen into the holocellulose (9) and would complicate the search for amide nitrogen from the liquid-ammonia treatment. Although chlorination in this way did not change the amide and ammonium nitrogen contents of the wood, subsequent extraction with dimethyl sulphoxide for 6 days at room temperature removed only 0.07% of the amide nitrogen which was insufficient to warrant large scale extraction.

Review of the literature showed that the carbohydrate fraction of wood, especially the hemicellulose, could be removed by the

following methods which could be considered comparatively mild:

(1) grinding of the wood in a vibratory ball mill followed by extraction with either dimethyl sulphoxide, dimethyl formamide or 50% aqueous acetic acid as developed by Björkman (70, 71, 72),

(2) extraction of wood with 3% aqueous solution of sodium sulphite (73), and

(3) extraction of the wood ground dry in a vibratory ball mill with water (74).

Method (1) is of limited utility, because considerable time is needed to extract only 15% of the carbohydrate material from wood. The second method was reported to have removed 28% of lignin and polysaccharide material from wheat straw and 14% of the same materials from oak at boiling temperature. When this method was applied to liquid-ammonia-treated white birch, 0.07% amide nitrogen was found in the extract and 0.17% amide nitrogen still remained in the residue. The third method was then tried. The liquid-ammonia-treated wood was ground in a National Bureau of Standards vibratory ball mill at 4° for 5, 20, and 45 hours without addition of liquid. The milled wood was then extracted with distilled water by boiling under reflux for 1 hour (wood: water = 1: 100). The aqueous extract and the residue were analyzed for nitrogen, with percentages shown in Table 3 based on oven-dry, liquid-ammonia-treated wood.

From these results it appeared that the amount of amide nitrogen extracted by hot water was a function of the duration of grinding and independent of the time of hot water extraction. The aqueous extract was clear and slightly yellowish when hot; it became turbid when it was allowed to cool to room temperature or concentrated to a small volume under reduced pressure at 50°. For this reason, the aqueous extract was

TABLE 3

Amide Nitrogen Contents of Aqueous Extract and Residue

Duration of Milling (hrs.)	% Extract	% Residue
0	0.03	0.22
5(a)	0.07	0.14
20	0.15	0.05
48	0.18	0.04

(a) Same result was obtained when the milled wood was extracted for 2 hours.

immediately filtered from the residue after 1 hour of reflux. Although the wood milled for 48 hours gave a slightly higher amount of amide nitrogen in the aqueous extract, the fineness of the milled wood caused considerable difficulty in subsequent filtration to separate the wood from the extracting liquor.

For preparative scale extraction, the ball mill was charged with 20.0 g. of the liquid-ammonia-treated wood, and ground for 30 hours. Extraction of the ground wood, followed by precipitation of the concentrated extract in ethanol gave a grey white powder in 22.2% yield. Analysis of the vacuum-dried hemicellulose showed it contained 0.53% amide nitrogen and 1.7% of Klason lignin. A small sample was hydrolyzed in acid and the hydrolyzate was chromatographed on paper with ethyl acetate-acetic acid-water and with n-butanol-pyridine-water as eluents. The movements of the major spots corresponded to those observed in control experiments with xylose, 4-O-methyl-D-glucuronic acid, 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose; only traces of galactose, glucose, mannose and arabinose were found. These results showed that the material extracted by hot water was a nearly pure 4-O-methylglucuronoxylan; it will now be referred to as "xylan amide"

CONFIRMATION OF THE AMIDE GROUPS IN ISOLATED "XYLAN AMIDE"

Since the isolated "xylan amide" contained a small amount of lignin (1.7%) contaminated with traces of other sugar, some confirmation of the amide groups actually derived from the uronic ester groups seemed mandatory.

The infrared spectrum of the isolated "xylan amide" (Figure 5, VI) retained the prominent amide I absorption band near 1,680 cm^{-1} already noted in the differential spectrum of the ammonolyzed wood from which the

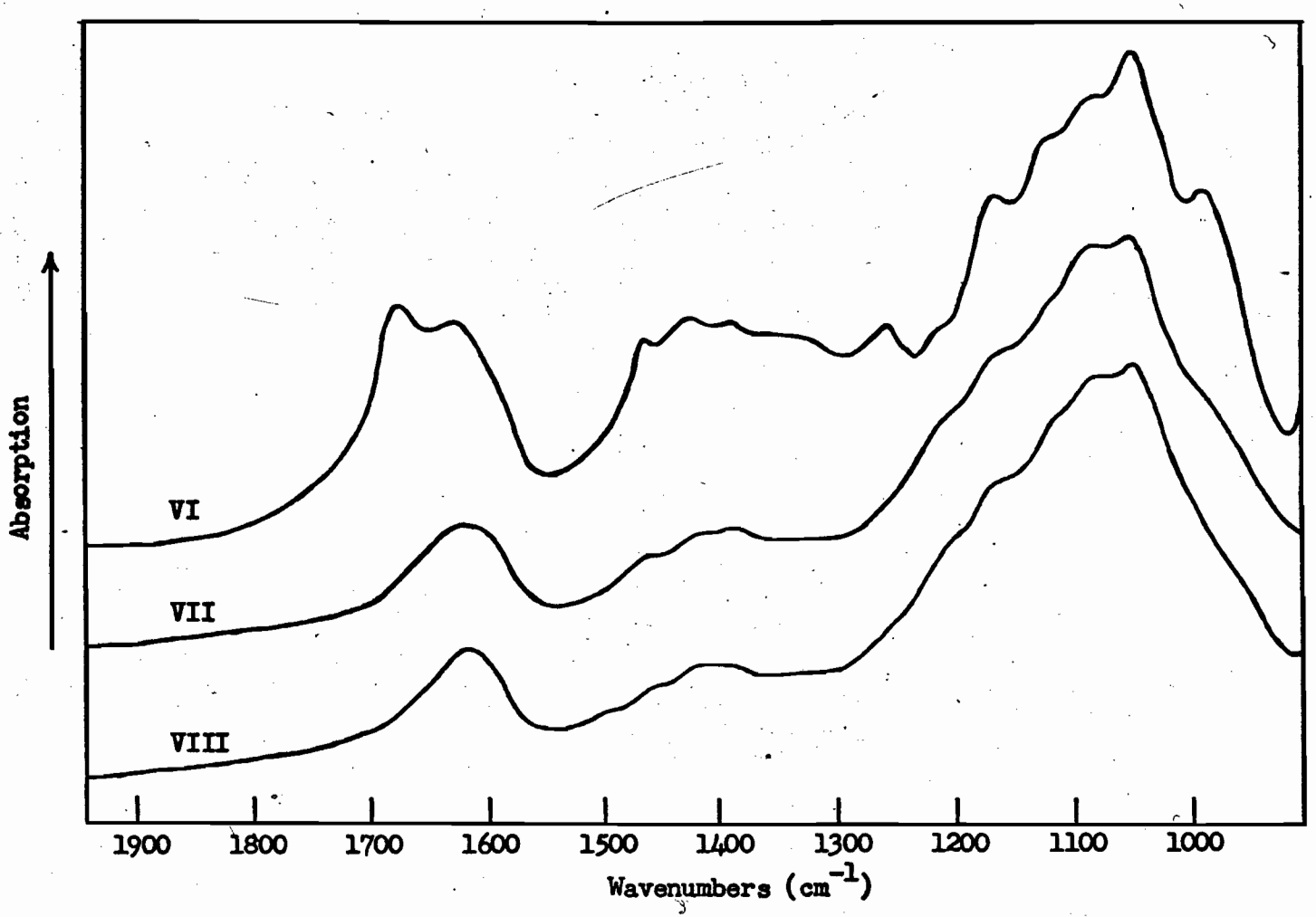


Figure 5 Infrared Spectra of "xylan Amide" from Birch Wood Treated with Liquid Ammonia (VI), "Xylan Amide" after saponification (VII), and Alkali-Extracted Birch Xylan (VIII).

xylan had been extracted (Figure 3, III). When this xylan was treated with 5% aqueous potassium hydroxide under nitrogen atmosphere, the amide band disappeared (Figure 5, VII) and the spectrum became identical with that of the xylan (Figure 5, VIII) extracted by 24% potassium hydroxide from untreated birch wood meal. The amide character of the nitrogen was further corroborated by the analytical results mentioned in the preceding section.

To show that the amide groups were not associated with any components of lignin, a sample of the isolated "xylan amide" was first dialyzed against distilled water for 24 hours and then subjected to partial enzymic hydrolysis using the Painter's method (31). A solution of the "xylan amide" and 0.2% of pectinase was enclosed in a cellophane bag and dialyzed for 24 hours against distilled water. The infrared spectrum (Figure 6) of the hemicellulose recovered from the dialyzate through precipitation with ethanol showed the prominent amide I band again at 1,680 cm^{-1} as noted previously. This hemicellulose contained no lignin, and, after acid hydrolysis, chromatography revealed only three major spots corresponding to 4-O-methylglucuronic acid, 2-O-(4-O-methylglucopyranosyluronic acid)-D-xylopyranose and D-xylose. Faint spots due to glucose and galactose were also noted.

These results further confirmed that the 4-O-methylglucuronoxylan had been isolated from the ammonolyzed wood as an amide.

ELIMINATION OF ALTERNATIVE INTERPRETATIONS

Simple consideration of the results presented so far led to the conclusion that the 4-O-methylglucuronamide groups of the xylan must have been formed by cleavage of ester links. Yet there was the clear possibility that these amide groups had arisen through other more complicated reactions of the carbohydrate materials with liquid ammonia.

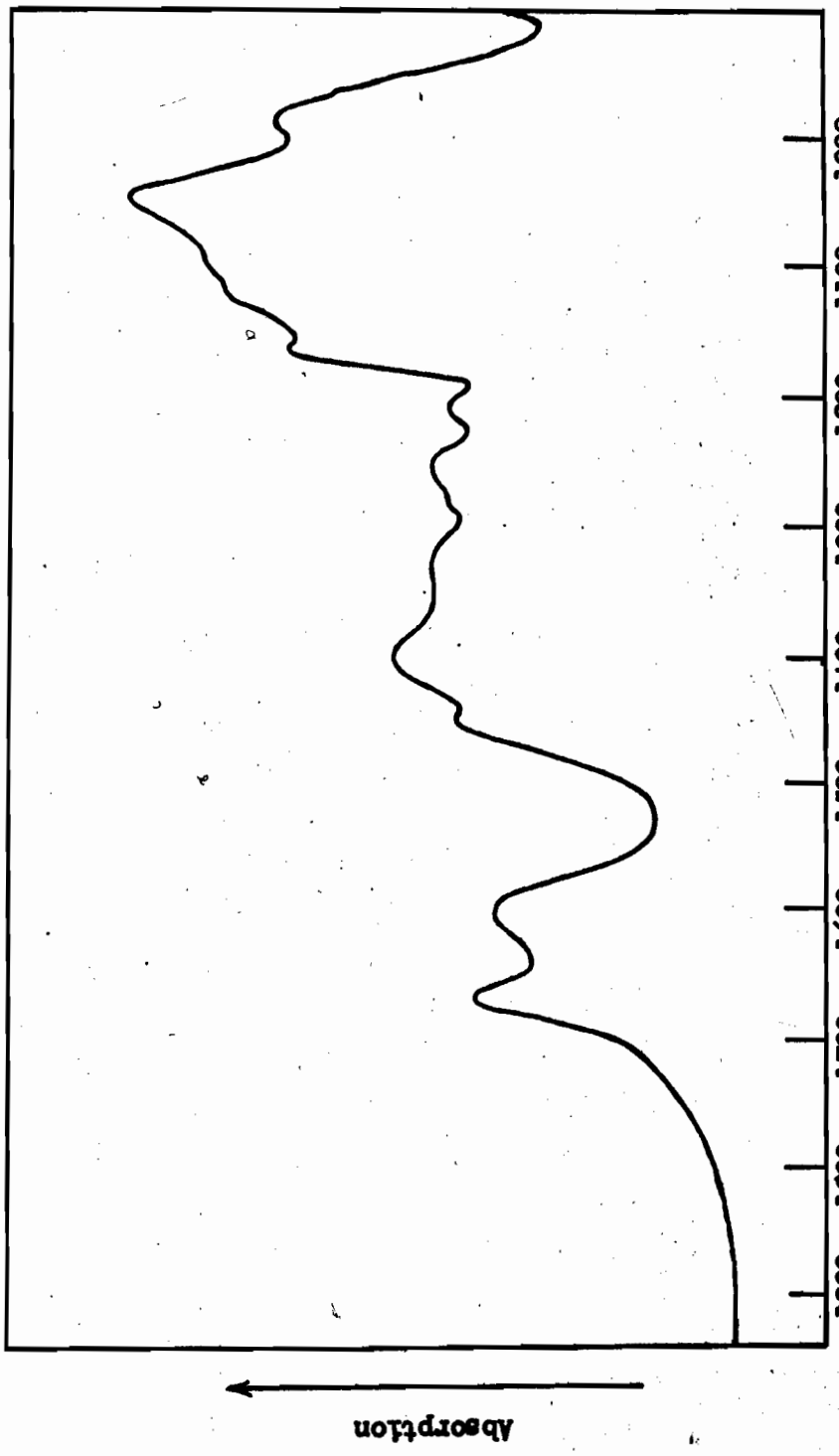


Figure 6 Infrared Spectrum of Enzyme-Hydrolyzed "Xylan Amide" from White Birch.

Although in 1934 Muskat (37) had claimed that mono- or polysaccharides could be recovered unchanged from liquid ammonia at -33°, Bishop (51) later reported the degradation of wheat straw hemicellulose, particularly polyuronides, in this reagent. In the patent literature (36), there was the suggestion that under higher temperature and pressure amides could even be formed from simple acids and liquid ammonia which probably involved the dehydration of the ammonium salt first formed. More recently, Hodge and Moy (75) had shown that simple reducing sugars react with liquid ammonia to form the corresponding glycosyldiamines. Deferrari and co-workers (76, 77, 78, 79) had isolated "aldobiose diamides" from the reaction of methanolic ammonia on acetylated aldobioses and proposed a mechanism for its formation based on the work of Isbell and Frush related to "aldose monoamide" (80). The intramolecularity of this mechanism was demonstrated by Deferrari (81) using labeled compounds.

Applied to the case of α-octa-O-acetylcellobiose (Figure 7, I), this mechanism involved the formation of a free aldehyde group (II) formed through ammonolysis of the acetyl group of the carbon atom 1. This first stage was followed by the formation of an intermediate (III) which would form the cyclic ortho ester (IV), in which the neighboring acetyl group participated. This cyclization was favoured by the polarization of the carbonyl group of the acetyl group rendering the carbonyl carbon atom electrophilic. The rearrangement of this ortho ester led to the formation of a N-acetyl derivative (V). The reaction could stop at this stage with the formation of N-acetylcellobiosylamine which was actually isolated from the reaction product. But most of the molecules having structure (V) would react with ammonia to give an intermediate, nitrogenated in the acetyl group of C-3 (VI), which would pass to the cyclic

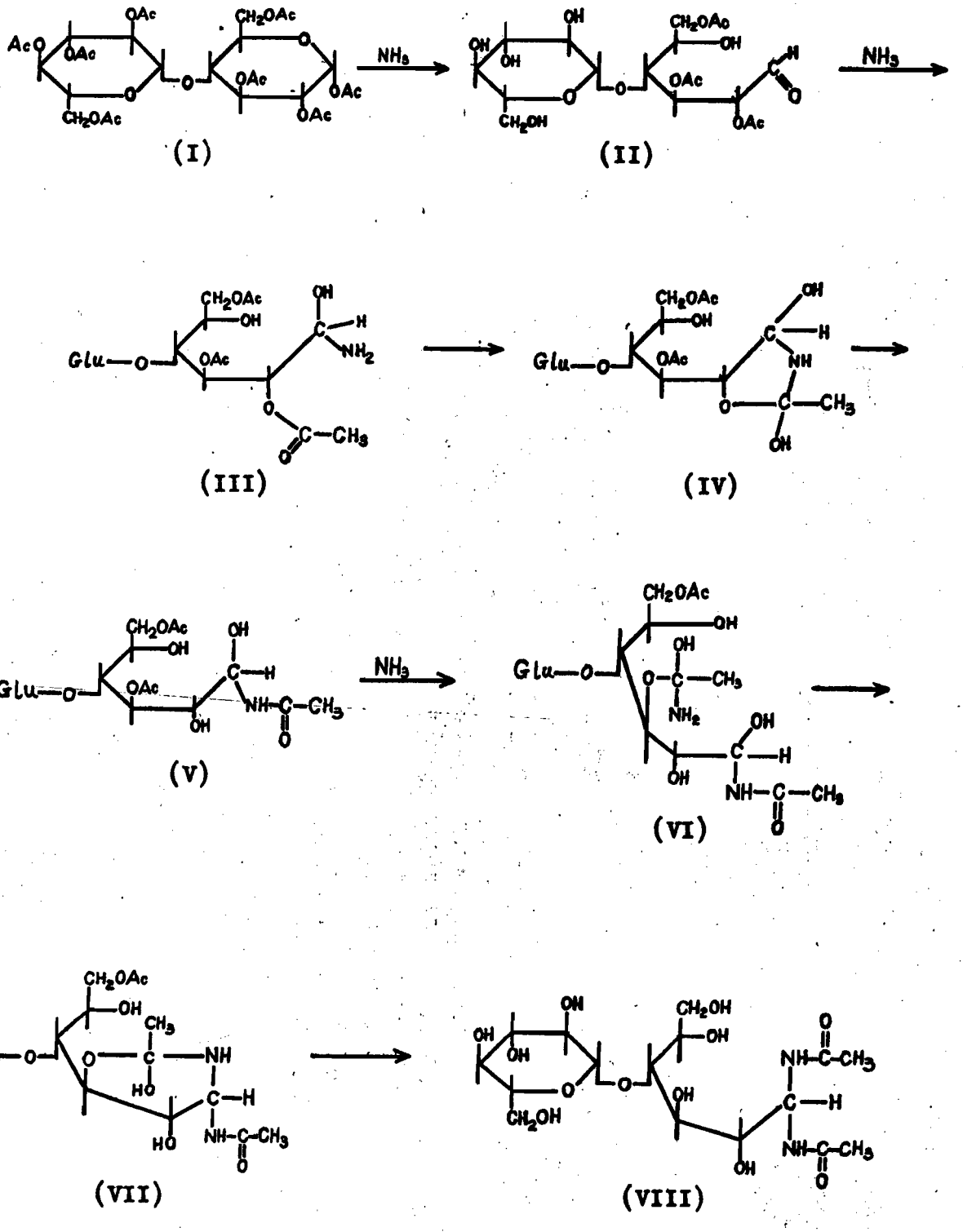


Figure 7 Mechanism for the Formation of Aldobiose Diamide

labile ortho ester (VII), affording finally the N,N'-diacetyl derivative on C-1 (VIII).

During the course of this ortho ester mechanism, another competitive reaction occurred, by which the remaining acetyl groups were split off. This was the known mechanism for the ammonolysis of esters and also occurred with the original β-octa-O-acetylcellobiose (I) affording free cellobiose. With acetylated disaccharides, the latter mechanism predominated leading to high yields of free sugar. The unusually poor yield (4%) of the "aldobiose diamide" compared with the yield (20 to 80%) obtained with acetylated monosaccharides was attributed (77, 78) to the steric hindrance exerted by the non-reducing moiety of the disaccharide upon the ortho ester mechanism.

The polysaccharide studied in the present work is the 4-O-methylglucuronoxylan whose structure can be represented as in Figure 8. This polysaccharide was shown by the classical methylation and hydrolysis techniques (82) to consist of a minimum of 110 β-D-xylopyranose residues linked together by 1,4-glycosidic bonds and with, on the average, every eleventh anhydroxylose unit carrying a single side chain of 4-O-methyl-D-glucuronic acid attached by an α-glycosidic linkage to C-2 of xylose. Recently, a renewed investigation of the 4-O-methylglucuronoxylan present in aspen wood (83) has led to the conclusion that other side chains carrying xylose units also occurred at C-3 of the xylan back-bone.

The 4-O-methylglucuronoxylan present in white birch was known to contain about 16.9% of acetyl groups (12). Bouveng (84) developed a reliable method for locating these acetyl groups in birch xylan and showed that this hemicellulose contained 58.1% unsubstituted, 11.8% 2-O-, 24.0% 3-O- and 6.1% 2,3-di-O-substituted xylose residues. In a separate

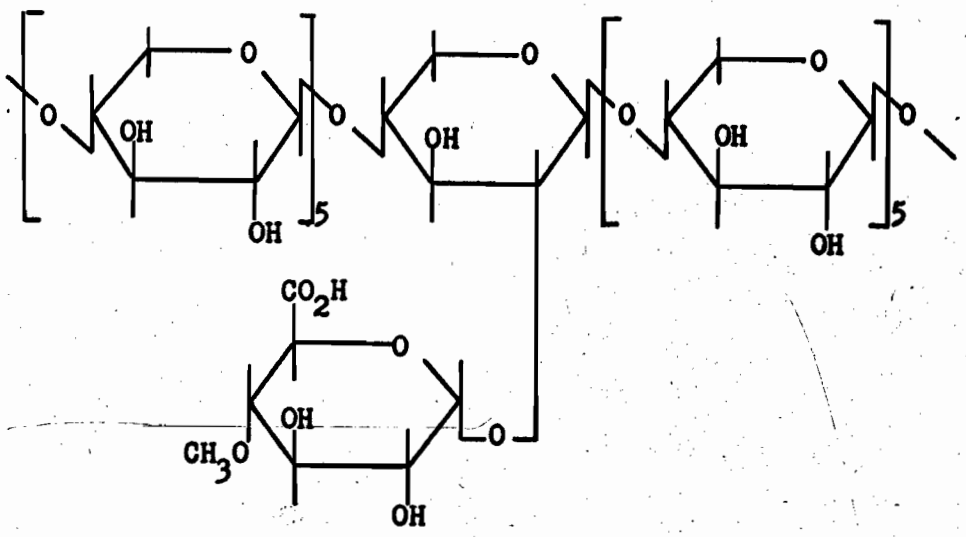


Figure 8 Simplified Structure of 4-O-Methyl-glucuronoxylan from White Birch

experiment, Timell (12) showed that the 4-O-methylglucuronic acid side chain carried no protecting acetate groups, because after a Smith degradation (85) of the polysaccharide, no uronic acid could be detected.

If treatment of white birch wood containing 24.6% acetylxylan with liquid ammonia resulted in "diamide" formation through the ortho ester mechanism, the conclusions based on analytical and infrared spectroscopic observations could be completely invalidated.

Some model experiments were, therefore, performed to investigate the possibilities just mentioned. On treatment of a fully acetylated cotton cellulose of $D.P._n$ 395 with liquid ammonia in the usual way, no "aldose diamide" was detected in the product. This observation supported the inference of Deferrari and co-workers (77, 78) that steric hindrance of the polymeric structure and the scarcity of the required reducing hemiacetal end-groups impeded the formation of "aldose diamide" in a polysaccharide. In addition, Hockett and Chandler (86) reported that "D-xylose diamide" could not be prepared from its acetyl derivative.

To determine whether acetamide derived from the ammonolysis of the acetylxylan might react with xylan, a mixture of 4-O-methylglucuronoxylan isolated from the same wood by alkali extraction and an authentic sample of acetamide was treated with liquid ammonia under pressure at room temperature as previously described. Infrared spectroscopy and quantitative amide nitrogen analysis of the xylan showed that no condensation between acetamide and xylan had occurred and formation of glycosylamine from ammonia with reducing end-groups also did not take place. Similarly, there was no conversion of the uronic carboxyl groups into amides by liquid ammonia under the experimental conditions employed.

Other control experiments included the treatment with liquid

ammonia of wood from which the xylan had been extracted with aqueous potassium hydroxide (24%), a reagent known to saponify all acetyl groups in the wood and presumably any other types of esters as well. The liquid ammonia treatment was also performed on a sample of extractive-free wood which had been reduced by sodium borohydride to effect the same total elimination of the acetyl groups possibly together with all the uronic ester groups originally present. As expected, both samples acquired ammonium, but not amide nitrogen. Similar results (Table 1, p.23) were obtained with the alkali-extracted xylan and this xylan in a mixture of 1: 3 with the "xylan poor" wood, and lent additional support to the view that intact ester groups were necessary precursors for the formation of uronic acid amides.

In a recent study of the pectic acids isolated from the bark of amabilis fir (Abies amabilis), Bhattacharjee (87) noted the shift of non-ionized carboxyl band from 1,716 cm^{-1} to 1,760 cm^{-1} in the infrared spectrum of D-galacturonic acid when it was recovered from an aqueous solution by evaporation of the solution in vacuo. Similar bands were also observed at 1,750 cm^{-1} in the infrared spectra of the polygalacturonic acids and were ascribed to the formation of lactone groups arising form drying or concentration of the solution in vacuo. An alkali-extracted xylan, after de-ashing and drying in the same way also showed a shift of the band from 1,610 cm^{-1} (due to ionized carboxyl groups) to 1,740 cm^{-1} in the infrared spectrum. In the literature (66, 85), the free carboxyl groups were reported to absorb at 1,725 cm^{-1}. The formation of lactone groups due to drying of the birch wood meal after solvent extraction before ammonolysis was then suspected. However, ammonolysis of the de-ashed xylan after drying over phosphorus pentoxide in

vacuo gave an infrared spectrum with only a band at 1,610 cm^{-1} indicating ionized carboxyl groups. No amide I band at 1,680 cm^{-1} was observed as in the "xylan amide" isolated from ammonolyzed wood. Further, analytical results showed the presence of ammonium nitrogen only and no amide nitrogen was found.

These negative results minized the possibility that the amide groups found in the 4-O-methylglucuronoxylan from ammonolyzed birch wood originated in some unsuspected side reactions, and strengthened the conclusion that many of the carboxyl groups in the untreated wood must have existed in the form of either lactone or ester groups, the latter as intramolecular cross-links within the xylan itself or between xylan and other wood constituents.

URONIC ESTER GROUPS IN OTHER NATIVE XYLAN

Confirmation of the presence of uronic ester groups in birch wood raised the question of whether these results might be considered general for other species. Such generalization need not be expected, because other xylans in the deciduous and coniferous woods have chemical compositions much different from that of white birch wood. It was also of interest to determine whether the method of isolating the "hemicellulose amide" could be more widely applied.

The other species investigated were one deciduous wood — trembling aspen (Populus tremuloides) and three coniferous — tamarack (Larix laricina), jack pine (Pinus banksiana), and balsam fir (Abies balsamea).

The xylan in aspen was reported in one instance (88) to be a linear framework of 212 xylose units, and on the average every ninth

anhydroxylose unit carried one 4-O-methylglucuronic acid. Other studies (83) showed that it was actually a branched polysaccharide involving short xylose side chains as well. However, it mainly retained the essential features of the xylan in other aborescent angiosperms in that the secondary hydroxyl groups at C-2 or C-3 were partly substituted with acetyl groups rather than L-arabinose units.

In the wood of the gymnosperms, the major hemicelluloses — arabinogalactan, glucomannan, and galactoglucomannan — have been the subject of many investigations. The minor hemicellulose — arabino-4-O-methylglucuronoxylan — has received much less attention. The structures of the xylan present in jack pine and balsam fir are still unknown, although the properties of other polysaccharides in these softwoods are now well established (85). From the available knowledge of other softwood xylans (85), it is expected that the xylan in jack pine and balsam fir must possess the general characteristics of arabino-4-O-methylglucuronoxylan common to all gymnosperms. An acidic xylan isolated in 12.9% yield from tamarack has been reported by Adams (89) to consist of 16 β-D-xylose units joined by a 1-4, or possibly 1-3, glycosidic linkage with side branches of three single units of 4-O-methylglucuronic acid attached through C-2 and one unit of L-arabinofuranose through C-3. Recent investigations (90) of the highly branched, water-soluble arabinogalactan present in the wood of tamarack have shown that this polysaccharide, contrary to previous results (91), contained, at the end of the chain, D-glucuronic acid groups attached to D-galactose units by β-(1-6) links.

To study the uronic ester groups in native xylans present in aspen, jack pine, balsam fir, and tamarack, the wood meals were first

exhaustively extracted with a benzene-ethanol mixture (2: 1), then with ethanol and finally with hot water (60°) for 24 hours to eliminate most of the water-soluble polysaccharide such as arabinogalactan known to be present in woods such as tamarack or maritime pine (90, 92). Treatment with liquid ammonia was applied for 72 hours as before to ensure the maximum amide nitrogen content. The ammonia-treated wood was then exhaustively extracted with methanol in a Soxhlet extractor to eliminate acetamide. The nitrogen contents of the wood were analyzed using the modified procedure already described. In all cases, the amide nitrogen contents in the treated wood (Table 4) are less than one-half or one-third of the value found for white birch (0.25%), though the uronic acid content of these woods are not much lower than the former. This might be regarded as the difference in the extent of esterification of the uronic acid groups in the woods so far studied.

The "hemicellulose amides" were then extracted by the ball-milling and hot water extraction procedure. The isolated polysaccharides were analyzed for ammonium and amide nitrogen contents as usual, with the results shown in Table 5.

Confirmation of the amide character of the nitrogen was again obtained through infrared spectroscopy. The infrared spectra (Figure 9) of the "hemicellulose amides" again showed the familiar amide I band at 1,680 cm^{-1} already noted for "xylan amide" isolated from liquid-ammonia-treated white birch. The amide I band appeared as a shoulder in the spectrum for the "hemicellulose amide" isolated from aspen wood. This was probably because of its lower amide nitrogen content (see Table 5) as compared to those from other woods.

After acid hydrolysis of the isolated "hemicellulose amides"

TABLE 4

The Ammonium and Amide Nitrogen Contents of Treated Wood

Wood	% Ammonium	% Amide	% Uronic Acid[a]
Aspen	0.14	0.08	3.3
Balsam Fir	0.16	0.10	3.4
Jack Pine	0.12	0.09	3.9
Tamarack	0.13	0.10	2.9

(a) Data of Timell (62).

TABLE 5

Nitrogen Contents of "Hemicellulose Amide" Isolated from Treated Woods

Wood	% Ammonium	% Amide
Aspen	0.05	0.21
Balsam Fir	0.13	0.32
Jack Pine	0.11	0.29
Tamarack	0.11	0.26

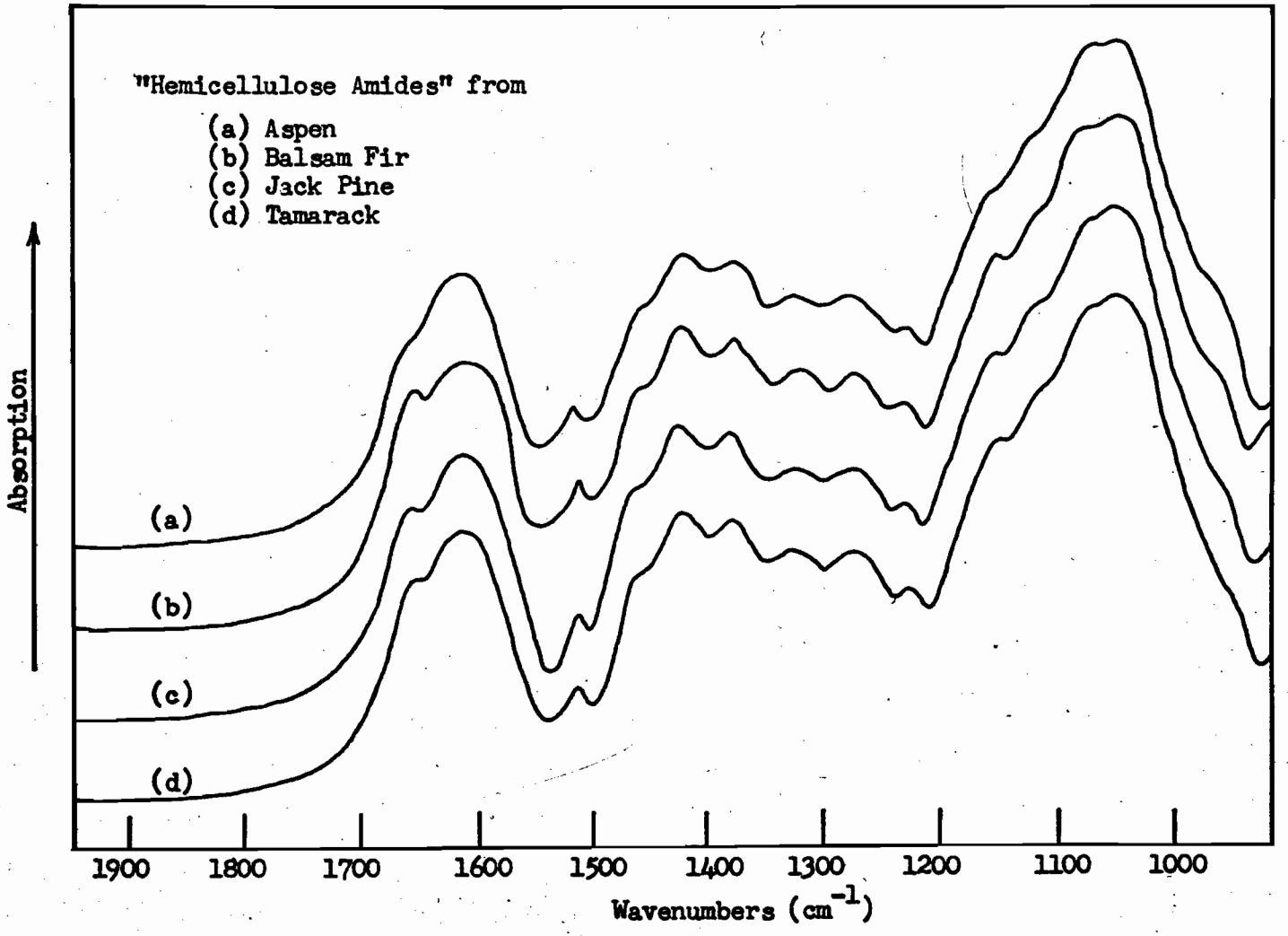


Figure 9 Infrared Spectra of "Hemicellulose Amides"

paper chromatography showed the presence of much xylose, uronic acid, mannose, and glucose, and only traces of other neutral sugars (Table 6). For a better identification of the uronic acids, they were separated from the neutral sugars by Dowex 1 X-4 anion exchange resin (acetate form), and their rates of movement on paper chromatograms were compared with authentic samples. The results showed that all the uronic acids were the well known aldobiouronic acid, 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose; no other sugar acids were found. The isolation of "arabinoxylan amides" from the softwoods further minimized the possibility of "diamide" formation due to acetyl migration through the intramolecular ortho ester mechanism already described (p. 38). The uronic acid amide groups present in the "hemicellulose amides" isolated from liquid-ammonia-treated aspen, balsam fir, jack pine, and tamarack were, therefore, derived solely from the esters of 4-O-methylglucuronic acid which occurred as side chains on the xylan back-bone.

TABLE 6

Sugar Components of "Hemicellulose Amide" After Acid Hydrolysis

Sugars Present	"Hemicellulose Amide" from Aspen	Balsam Fir	Jack Pine	Tamarack
Uronic Acid	+	+	+	+
Galactose	trace	trace	trace	trace
Glucose	trace	+	+	+
Mannose	++	++	++	++
Arabinose	trace	trace	trace	trace
Xylose	+	++	++	++

EXPERIMENTAL

MATERIALS

Wood meals, 60 to 80 mesh, from white birch (*Betula papyrifera*), aspen (*Populus tremuloides*), tamarack (*Larix larcina*), jack pine (*Pinus banksiana*), and balsam fir (*Abies balsamea*) with unknown history and background were first exhaustively extracted separately in a Soxhlet extractor for 36 hours with 1: 2 azeotropic solution of ethanol and benzene. This treatment was followed by extraction with hot ethanol for 24 hours. The bag of meal was then removed from the Soxhlet extractor, and hot (60°) tap water was run through the meal for 48 hours. After a 4-hour soaking in distilled water, the meal was air-dried for 3 days, rescreened to separate the 60 to 80 mesh portion and stored in screw-capped amber bottles so that the moisture content would remain roughly constant over long periods.

A published method (93) was used to extract 4-O-methylglucuronoxylan with 24% aqueous potassium hydroxide from extractive-free white birch wood meal; yield: 24.3%. Acid hydrolysis followed by paper chromatography (94) indicated the presence of much xylose and only traces of other sugars. The resulting "xylan poor" wood residue (75.2%) was washed with distilled water until neutral to universal indicator paper, next with 0.02 N hydrochloric acid, then with distilled water again to neutrality, and, finally air-dried.

Commercial liquid ammonia of 99.9% purity was used according to the manufacturer's specifications.

The acetylated raw coastland cotton cellulose used in control experiments was described by Timell (95).

Dioxane lignin was prepared according to the procedure of Pepper and co-workers (65).

ANALYTICAL PROCEDURES

The following analyses were carried out according to the procedure described in "Testing Methods of the Technical Association of the Pulp and Paper Industry, New York, N. Y. (1961)":

Klason Lignin Content	T 13m - 54
Ash Content	T 15m - 58
Moisture Content	T 210m - 58
Holocellulose	T 9m - 58

TOTAL NITROGEN

Total nitrogen was determined on 1.0 g. samples by an adaptation of the Gunning method (96). Results were reported as percent elementary nitrogen calculated on oven-dry basis.

AMMONIUM AND AMIDE NITROGENS

A standard method (59) was adapted as follows for the determination, first of ammonium and then of amide nitrogen. An air-dry, 1.0 g. sample was placed in a 300 ml., 3-necked round-bottom flask equipped with 250 ml. dropping funnel and a U-shaped splash-head connected to a condenser; the third neck was stoppered. The lower, distal end of the condenser was immersed in 25 ml. of 1% aqueous boric acid containing 2 drops of a methyl purple indicator and just enough 0.02 N hydrochloric acid to make the solution purple. A suspension of 5 g. of magnesium oxide (A.R. Grade) in 150 ml. of water was added through the dropping funnel, and the mixture

was heated until about 80 ml., containing all of the liberated ammonia, had distilled into the boric acid solution. The latter was then titrated with 0.02 N hydrochloric acid, and the percentage of ammonium nitrogen was calculated. After providing a fresh 25 ml. volume of the boric acid, with the indicator and of the proper pH, 150 ml. of 60% aqueous potassium hydroxide was added through the dropping funnel and the third neck of the flask was equipped for a steam distillation; an ordinary distillation caused excessive "bumping". About 80 ml. of distillate was collected in the boric acid solution which was then titrated as before. The result corresponded to the content of amide nitrogen, when correction was made for the small blank. On wood samples, moisture content was first determined on a separate sample and the nitrogen values were reported on oven-dry basis. Calculation:

$$\% N = \frac{(V_2 - V_1) \times N \times 14}{W} \times 100\%$$

Where

V_1 = blank

V_2 = volume of HCl equivalent to ammonia, ml.

N = normality of HCl

W = weight of sample in mg.

Control experiments with pure samples or known mixtures of acetamide and ammonium acetate showed the method to be reliable. Found for pure acetamide, m.p., 81.5°; amide N, 23.2, 23.6; calc.: 23.8. Found for pure ammonium acetate: ammonium N, 17.8, 17.8; calc.: 18.2. Found for a mixture containing 0.30 g. of acetamide and 0.63 g. of ammonium acetate: amide N, 7.6; ammonium N, 11.6; calc.: amide N, 7.7; ammonium N, 12.3. For a mixture containing 0.51 g. of acetamide and 0.46 g. of ammonium

acetate, found: amide N, 11.9; ammonium N, 8.3; calc.: amide N, 12.1; ammonium N, 8.4.

PAPER CHROMATOGRAPHY

Solvent systems used for paper chromatographic resolution of sugar mixtures were (in vol./vol.):

(A) ethyl acetate- acetic acid-water (9: 2: 2)

(B) n-butanol-pyridine-water (10: 3: 3)

All separations were carried out on Whatman No. 1 filter paper by the descending technique. The chromatograms were developed for 24 hours when using solvent (A), and 34 hours when solvent (B) was used. The spray reagent consisted of a solution of ortho-aminodiphenyl (3.0 g.) and 85% phosphoric acid (1.3 ml.) in glacial acetic acid (100 ml.) (97). The paper was sprayed evenly and heated in an oven at 105° for 2 to 4 minutes. Hexoses appeared as brown spots, pentoses appeared as red spots, and monouronic acids appeared as orange spots which turned violet upon further heating. Finally, aldobiouronic acid appeared as orange spots initially, changing to brick-red upon prolonged heating.

ACID HYDROLYSES OF HEMICELLULOSE

Samples of the hemicellulose (1-0.5 g.) were hydrolyzed by treatment with 72% sulphuric acid (5 ml.) for 2 hours, at room temperature, followed by dilution to 180 ml. and boiling under reflux for four to six hours. The hydrolyzate was neutralized to a pH of 8 with solid barium carbonate and filtered; excess barium ions were removed with Amberlite IR-120 ion-exchange resin. The solution was evaporated to 3 ml., and subjected to paper chromatography (solvents A and B).

INFRARED SPECTRA

A Unicam S.P. 100 prism-grating spectrophotometer was used to determined infrared spectra. The samples were ground in a stainless steel vial for 5 minutes, then mixed with the desired amount of potassium bromide (usually 400 mg. KBr to 1.6 mg. of sample), milled for additional two minutes and finally transferred into a die to be pressed into discs 16 mm. in diameter. Differential spectra of holocellulose in birch wood were determined on samples of known Klason lignin content. An amount of birch dioxane lignin corresponding to the Klason lignin content of the wood samples was incorporated in a similar disc for use in the reference beam.

Since only 1.6 to 4.5 mg. of sample was required in each 400 mg. disc, to ensure each disc having the prescribed amount of material, an 100 mg. excess of the mixture containing the sample and potassium bromide was prepared. Exactly 400 mg. of the mixture was then carefully weighed out on an analytical balance and transferred quantitatively to the die. The die was evacuated, and, after 5 minutes, pressure was applied (20 tons on the ram). The pressure was maintained for 10 minutes, then released, and finally the vacuum was broken. The disc was then carefully removed from the die by forceps.

SMALL-SCALE AMMONOLYSES

Small-scale ammonolyses were carried out at room temperature for various times in a bomb consisting of a stainless steel cup of 280 ml. capacity (A in Figure 10), closed by a lid held in place by means of a bolt threaded through a heavy steel yoke. A polyethylene gasket between the cup and the lid prevented any leakage of ammonia. In a typical

Figure 10 Stainless Steel Bombs for Liquid Ammonia Extractions

experiment, a 10.0 g. sample of the solvent-extracted birch wood meal was added to the bomb which was then cooled to -60° in dry ice and acetone mixture with its lid sitting loosely in position. At the same time, approximately 200 ml. of liquid ammonia was collected from a cylinder in the glass flask (A in Figure 11) also cooled in dry ice-acetone. Approximately 150 ml. of liquid ammonia was poured into the bomb to make a wood to ammonia ratio of 1: 15. The lid was replaced, the bomb placed in the yoke and the bolt tightened with a wrench. The filling operation required less than 3 minutes after the bomb had cooled, and was carried out in a well ventilated fume cupboard. The bomb was then mounted on a mechanical shaker.

The apparatus (Figure 11) used in emptying the bomb and in filtering the liquid ammonia extract was a modification of that used by Sanderson (98). At the end of a desired reaction time, the bomb, still in the yoke, was quickly cooled in a dry ice-acetone bath, care being taken that the level of the cooling mixture remained an inch below the gasket in order to prevent any contamination of the contents with acetone.

The bomb was then removed from the cooling bath, the bolt slackened slightly, and the lid gently tapped several times on each side with a hammer. This precaution was found to be necessary in order to free the gasket without damage. The bolt was then completely loosened, the bomb removed from the yoke and the lid lifted off. The cup was attached to the rubber stopper (B in Figure 11) fitted with a Pyrex filter stick outlet and a short glass tubing inlet. A water aspirator was used to suck the liquid ammonia extract into flask (C) and the side tube (D) was then closed by clamping. Fresh liquid ammonia (500 ml.), was run in from reservoir (E) to wash the residual wood. The towers of solid

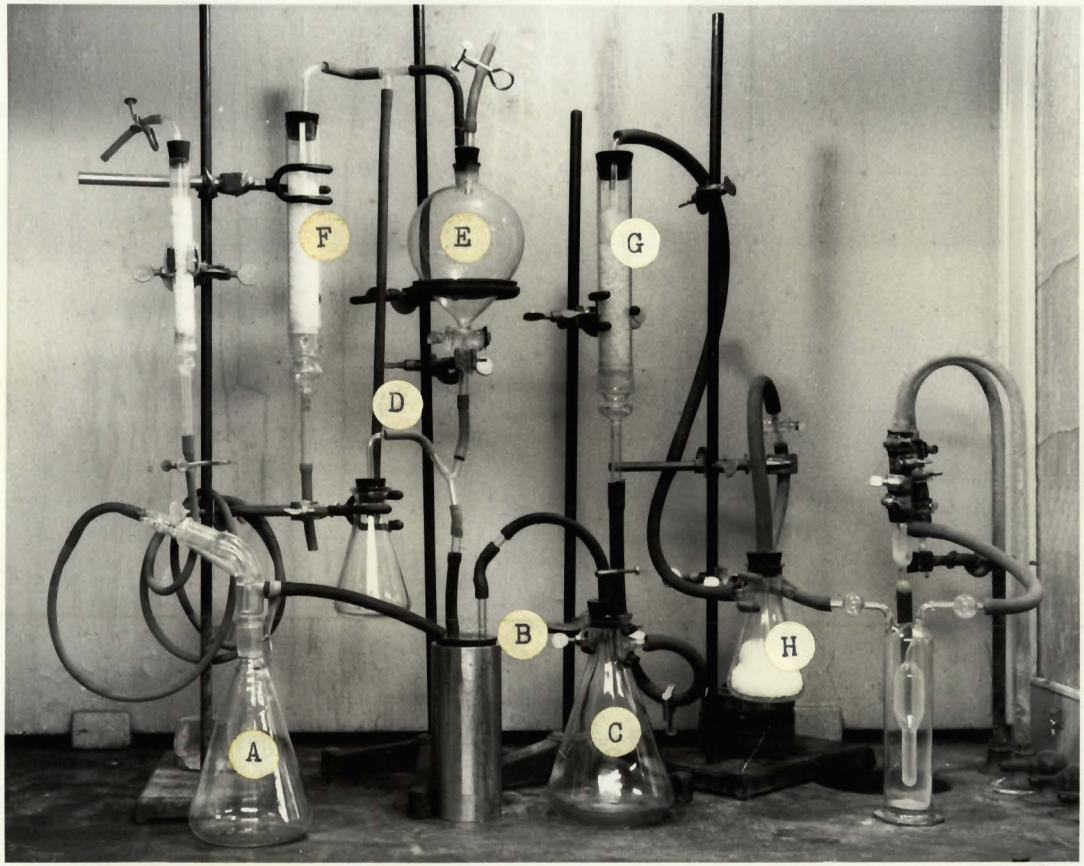


Figure 11 Assembly for Discharging Liquid Ammonia from Large Bomb

potassium hydroxide (F, G, H) served to protect the system from moisture and to prevent the build-up of a dangerous pressure in the system. When washing was complete, the liquid ammonia in flask (C) was evaporated under continuous suction of the water aspirator. A 1-hour ammonolysis extracted 3.9% of the wood, and a 100-hour treatment removed only 4.6%. The liquid ammonia extract contained mostly acetamide with small amounts of lignin and polysaccharide.

The ammonia remaining in the wood was removed by keeping the sample under vacuum for 12 hours. The wood was extracted in a Soxhlet apparatus with methanol for 2 hours to remove entrained acetamide. After drying in air, the sample was analyzed for nitrogen, and the results shown in Table 1 were the means of concordant duplicates.

In control experiments using material such as alkali-extracted xylan from white birch, the lid of the bomb was loosened slightly at room temperature by slackening the bolt at the end of the reaction. The bomb, still in the yoke, was placed immediately into a vacuum desiccator containing solid potassium hydroxide. Liquid ammonia in the bomb was allowed to evaporate under a water pump.

LARGE-SCALE AMMONOLYSES

Large-scale ammonolyses were carried out for 72 hours to ensure maximum conversion of esters into amide groups. Yields of wood meal after ammonolysis were shown in Table 7.

SODIUM BOROHYDRIDE REDUCTION OF BIRCH WOOD

Ten grams of birch wood was suspended in 150 ml. of distilled water containing 7 g. of sodium borate, and sodium borohydride (7 g.)

TABLE 7

Yields of Wood Meal After Ammonolyses

Wood	% Yield (oven-dry basis)
Birch	91.8
Aspen	95.0
Jack Pine	93.8
Balsam Fir	92.9
Tamarack	92.5

was added in small proportions over a period of 2 hours. Additional 7 g. quantities of the hydride were added each day for 4 days, and the suspension was stirred mechanically at room temperature for 10 days. Excess sodium borohydride was destroyed by the addition of acetic acid (30% in water), and the reduced wood meal was recovered by filtration and washed thoroughly with distilled water. The air-dried meal was almost white in appearance; yield: 9.1 g. Acetyl content was zero; liquid ammonia treatment of the reduced wood gave an amide nitrogen content of 0.01%.

PREPARATION OF α-D-GLUCURONAMIDE

In a modification of methods described in the literature (99, 100), 5 g. of glucuronolactone was dissolved in 150 ml. of anhydrous methanol saturated with ammonia at 0°. The solution was kept in the cold room at 4° for 5 days, until a white material (1-amino-D-glucuronamide) had been deposited on the sides of the flask. After filtration and drying over phosphorus pentoxide *in vacuo*, the yield of crude product was 2.6 g. One gram of the unpurified amino-glucuronamide was dissolved in 150 ml. of water and passed through a column of Amberlite IRC-50 (H^+) resin in a 100 ml. buret (resin column height up to the 80 ml. mark). The eluent was concentrated to 40 ml. under reduced pressure, and 200 ml. of a mixture of methanol and isopropanol (1: 1, v/v) was added. The white precipitate was recovered, and recrystallized 5 times from water-isopropanol; yield: 0.78 g.; m.p., 153-4° (dec.); reported, 150° and 158°; $[\alpha]_D^{20}$ (2% in water), +46° (10 min.); +31° (90 min.); +29.3° (22 hours); reported (99): +78° (3 min.); +31.6° (22 hours). Calc. for $C_6H_{11}O_6N \cdot H_2O$: N, 6.63; found: N, 6.47, 6.51.

ATTEMPTED ISOLATION OF "XYLAN AMIDE" BY ENZYMIC HYDROLYSIS

A 1.0 g. sample of liquid-ammonia-treated white birch wood was suspended in 20 ml. of a 10% aqueous solution of pectinase (supplied by Nutritional Biochemical Co., Cleveland, Ohio). The solution was buffered to pH 4.6 and slowly rotated at 40° in a 25 ml. screw-cap vial, toluene being used as a preservative. At the end of the reaction period (4 days), the solution was transferred into a cellophane bag and dialyzed against distilled water to separate the hydrolyzate. However, the concentrated enzyme solution enclosed in the bag acted on the cellophane membrane and caused it to rupture in 2 hours. Attempts were then made to precipitate the enzyme before dialysis by boiling the solution for 1 hour as recommended by the supplier of the enzyme. However, boiling only resulted in a highly viscous brown solution with no sign of precipitation. This method was then discarded.

ISOLATION OF THE AMIDE OF 4-O-METHYLGLUCURONOXYLAN

After the residual wood from ammonolysis had been extracted with methanol and dried in air, 20 g. was ground with no addition of liquid in a vibratory ball mill (101) for 30 hours at 4°. The mill was powered by an 1,800 rpm 1/4 hp electric motor which drove the mill shaft and attached eccentric weight (A in Figure 12). Rapid rotation of the weight imparted a revolutional motion to the jar (B) when mounted on its holder (C) which was suspended by two strong springs (D), thus allowing freedom of circular movement within certain limits. Thirty-five hundred tiny steel balls in the jar were set into rapid motion by this movement and ground the material placed in the jar by their collision.

The finely divided product was separated from the balls by screening and was then boiled for 1 hour under reflux with 2 litres of

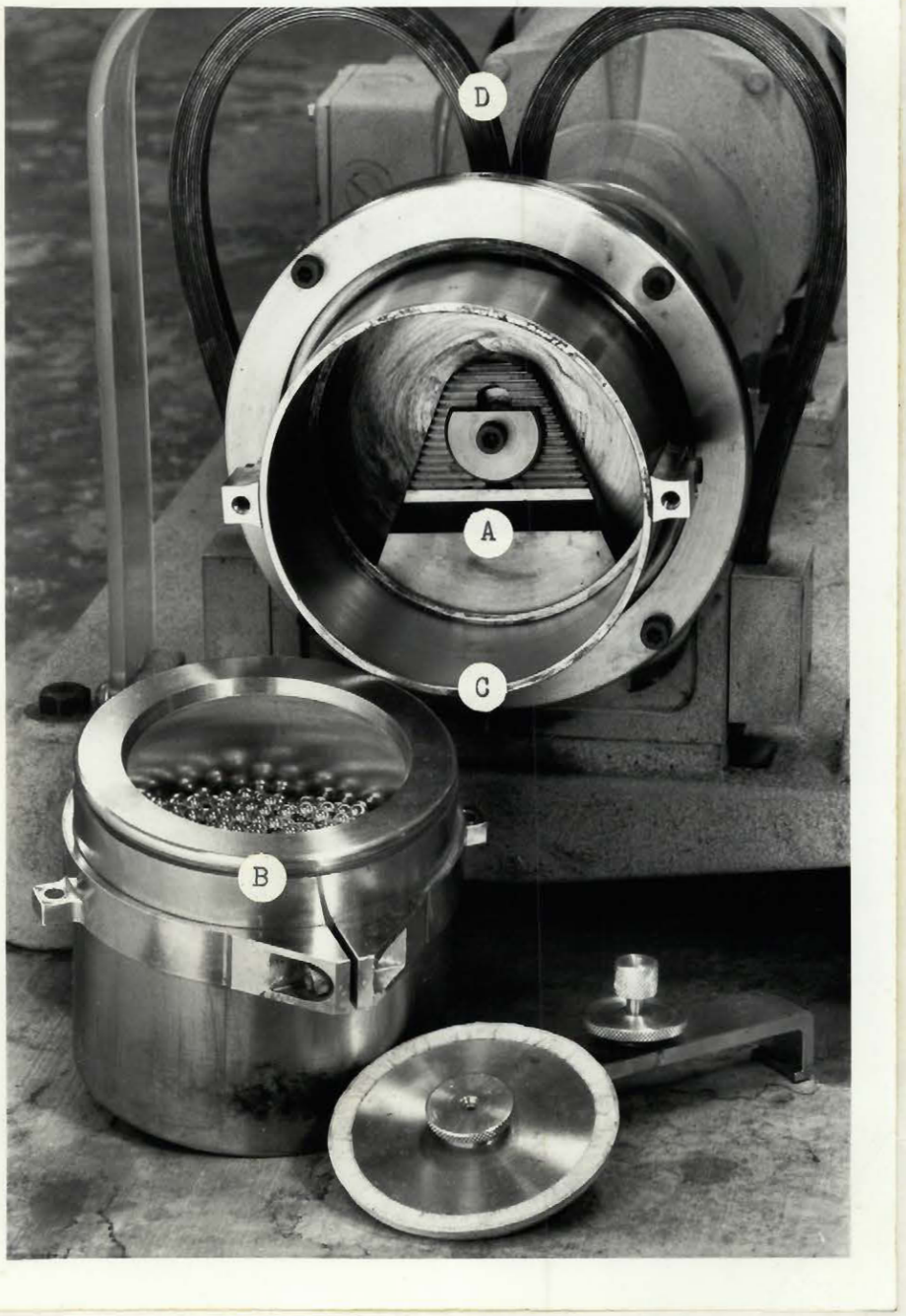


Figure 12 Vibratory Ball Mill

distilled water. Without cooling, the suspension was filtered through a medium porosity, sintered-glass funnel, and the residue was washed with three 500 ml. volumes of boiling distilled water, then with 500 ml. of cold water. The combined liquors were concentrated at 50° under reduced pressure to about 70 ml., the concentrate was poured, with vigorous stirring, into 2 litres of ethanol, and the mixture was kept overnight near 4°. The precipitate of hemicellulose was then recovered on a filter, solvent-exchanged through ethanol and petroleum ether (b.p. 30-60°) and dried in vacuo. Acid hydrolysis followed by paper chromatography showed that this hot-water-extracted material was a nearly pure xylan. The yields of the hemicellulose isolated this way from different woods are presented in Table 8.

In a smaller-scale experiment, 1.0 g. of the finely ground wood from liquid-ammonia-treated birch was extracted as before with hot water, the amide nitrogen contents of the extract and the residue were 0.15% and 0.05%, respectively, on the wood basis. That is, hot water had extracted 75% of the amide nitrogen from the liquid-ammonia-treated wood, and it was in the xylan.

ALKALI HYDROLYSIS OF "XYLAN AMIDE" FROM WHITE BIRCH

A 100 mg. sample of the hemicellulose isolated from liquid-ammonia-treated birch wood meal was boiled under reflux in a nitrogen atmosphere for 3 hours with 10 ml. of 5% potassium hydroxide. The alkali was neutralized with acetic acid and the hemicellulose was recovered by precipitation in ethanol; yield: 92 mg. This treatment removed the amide I band at 1,680 cm^{-1} from the infrared spectrum (Figure 5, VII).

TABLE 8

Yields of "Hemicellulose Amide"
from Different Woods

Wood	% Yield	% Klason Lignin
Birch	22.16	1.70
Aspen	19.50	5.25
Balsam Fir	19.03	3.62
Jack Pine	19.63	2.85
Tamarack	20.45	3.38

PARTIAL ENZYMIC HYDROLYSIS OF THE "XYLAN AMIDE" FROM WHITE BIRCH

"Xylan amide" (1.4 g.) was first dialyzed against distilled water for 24 hours, and then added to 25 ml. of purified pectinase solution containing 0.14 g. of the enzyme. The final volume of the solution was adjusted to 70 ml. so as to attain a concentration of 2% xylan in a 0.2% aqueous solution of the enzyme. The solution was enclosed in a cellophane dialysis tube of such length so as to permit a 100% increase in volume of the solution by osmosis. The dialysis tubing was suspended in water between two perforated desiccator plates inside a 3-litre beaker, with the lower plate resting on four rubber stoppers and the upper plate kept in position with wires. Two litres of water was placed in the beaker, and stirred vigorously at room temperature. After 8 hours, the dialyzate was withdrawn and replaced with fresh water, and this procedure was repeated for a total time of 24 hours. The combined liquors were evaporated under reduced pressure at 40° and the corresponding oligosaccharides were recovered through precipitation with ethanol; yield: 0.2 g., Klason lignin content, 0. An infrared spectrum of the material again showed the prominent amide I band at 1,680 cm^{-1} as the original "xylan amide". Acid hydrolysis followed by paper chromatography (solvent A) revealed the presence of 4-O-methylglucuronic acid, 2-O-(4-O-methylglucopyranosyluronic acid)-D-xylopyranose, and D-xylose; traces of glucose and galactose were also found.

PART III

INTERMOLECULAR BONDS: PREPARATION AND ANALYSIS OF "LIGNIN-CARBOHYDRATE COMPLEXES"

INTRODUCTION

ISOLATION OF XYLAN WITH INTACT ESTER GROUPS

The evidence in the previous Chapter has clearly established that a significant proportion of the carboxyl groups in native hemicelluloses is esterified, but has revealed nothing of the source of the hydroxyl groups involved in the ester linkage. To approach this problem, it required the isolation of a xylan with intact ester groups. Insofar as the retention of acetyl groups might be a measure of this requirement, several possibilities could be considered

The first recorded isolation of xylan containing native acetyl groups was made by Hägglund and co-workers (102) who extracted chlorite holocellulose with dimethyl sulphoxide. They obtained the xylan in 5% yield — a proportion not high enough to be considered representative. Aside from the low yield, this xylan may not be useful for present purposes, because the glucuronic ester groups may have been altered as indicated by the migration of acetyl groups during the preparation of chlorite holocellulose. Better yield of the acetyl xylan has been obtained by extracting birch chlorine holocellulose with dimethyl sulphoxide (12). However, this xylan is also not a suitable starting material for the study of uronic ester cross-links by the ammonolysis reaction, because the retention of nitrogen by the holocellulose (9) probably indicates that the uronic ester groups might have been altered by the hot alcoholic monoethanolamine solution used in the removal of chlorolignin during delignification.

Marchessault and co-workers (52) recently showed that hot water

extracted a substantial amount of acetyl xylan from chlorite holocellulose if the latter had been first treated with liquid ammonia or ethylamine for a short time (30 seconds). However, this prerequisite swelling of the holocellulose by nitrogen compounds is again objectionable.

It is clear that in the required method of isolation any treatment with reagents, even in the mildest delignification procedures, is to be avoided. The best way is to isolate the xylan from whole wood with neutral solvents. To this end, the advantages of extracting milled wood may be considered.

INCREASE IN SOLUBILITY OF WOOD COMPONENTS BY MILLING

Studies on the effect of milling on the solubilization of wood components arose from the work of Forziati and co-workers (101) on cotton cellulose. Their results indicated that milling caused negligible oxidation of the cellulose, but resulted in marked decrease in the degree of polymerization and at the same time converted the cellulose completely from a crystalline to an amorphous form. Grohn and Schierbaum (74) investigated the milling of wood, and reported that about 34% of milled spruce or poplar wood became soluble in cold water. The residual wood after extraction was easily susceptible to the action of cellulytic enzyme and subsequently an additional portion of the enzyme-degraded wood became soluble in cold water. Altogether, 54% of the wood was rendered soluble by the combined action of milling and enzymic hydrolysis.

Pew (68) studied the solubility of milled spruce wood in other solvents. A marked increase in solubility was observed in dilute alkali, acids, and a variety of organic solvents such as alcohol, acetone, pyridine,

methyl cellosolve, and dimethyl sulphoxide. Even periodate and Klason lignins, after they were milled for 5 hours, became soluble in dilute alkali and methyl cellosolve.

Brownell and West (103) have shown that milled spruce wood has a high degree of chemical reactivity. They treated the wood powder with ethylene oxide in dimethyl formamide at 100°, and found that within 3.5 hours, the wood became completely soluble in the solvent.

However, the most promising approach to the isolation of an acetyl xylan probably still retaining the uronic ester groups was a by-product of the method developed by Björkman (71) primarily for the extraction of lignin. After "milled wood lignin" had been extracted from finely divided silver birch wood by dioxane, further extraction of the residue with aqueous acetic acid (50%) or dimethyl sulphoxide for almost 11 months yielded a grey-white material which Björkman called a "lignin-carbohydrate complex". It contained 13% of Klason lignin, 75% of acetyl xylan, and small amounts of other carbohydrates, all present in the original wood (Table 10). It thus seemed possible that such a complex might be a useful material for the study of uronic ester cross-links.

ELECTROPHORESIS OF THE COMPLEX

Proof that the carbohydrate and lignin are joined by chemical rather than physical bonds is not easily obtained. The shortcomings of conventional methods of fractional precipitation in the separation of different polysaccharides (24) and lignin containing mixed hemicelluloses (104) have been recognized. The only reliable method for achieving complete physical separation of lignin and polysaccharide seems to be zone electrophoresis.

The use of zone electrophoresis in the separation of simple sugars and polysaccharides has received much attention in recent years (105). Many polysaccharides hitherto assumed to be essentially homogeneous have been further separated into different components (106), but only a few isolated examples of the use of boundary electrophoresis on lignin have been mentioned (107).

The supporting matrix most commonly used in zone electrophoresis is filter paper, although other materials such as starch, silica gel, and glass-fibre paper have found some application, and in certain cases may be more advantageous than filter paper. The operation of zone electrophoresis involves the application of an electric field across a strip of paper, impregnated with a suitable conducting solution, onto which a small amount of the substance (5-150 μg.) under examination has been introduced as an arbitrarily located, compact zone. After a convenient interval of time, the paper strip is processed in much the same manner as in standard paper-chromatographic procedure. The position of the zones may be ascertained by making use of specific physical properties (for example, UV absorption) or chemical reactivity (for example, reducing power) of the migrated substance.

Since in zone electrophoresis, the impregnated paper strip is the effective resistance in an electrical circuit, heat will be generated within its structure. Ineffective or incomplete dissipation of this heat will lead consecutively to evaporation of the conducting solution, establishment of a concentration gradient, and ultimately, the breakdown of the circuit if a dry zone is formed across the paper strip. The most versatile device currently used in zone electrophoresis is the enclosed-strip apparatus which comprises a paper strip pressed between glass plates on a metal cooling

plate. The ends of the strip are projected beyond the edges of the glass plates and dipped into the electrode chambers. Temperature regulation is effected by conduction through the glass plates. The application of high potential gradients has two distinct advantages in zone electrophoresis. First, a rapid separation of the components of a mixture may be permitted. This is of considerable importance in routine work. Second, diffusion effects, which may interfere with the resolution of mixtures of substances having closely similar mobilities, may be eliminated.

Lindgren (115) has been the only one so far to apply high voltage glass-fibre paper electrophoresis to a material containing both lignin and polysaccharide previously isolated by Björkman from milled spruce wood (71). The spruce "milled wood lignin" and a lignin fraction extracted from the "lignin-carbohydrate complex" were shown to exhibit the same electrophoretic movement relative to hydroxymethyl furfural, while the "lignin-carbohydrate complex" could be separated by this technique into two sub-fractions. The slow-moving fraction was entirely carbohydrate and the other fraction contained both lignin and carbohydrate. These results provided the first acceptable physical evidence supporting the presence of a covalent lignin-carbohydrate bond in an isolated "complex".

However, it is known (108) that under milling conditions, free radicals are generated together with breaking and formation of covalent bonds. Since phenolic compounds like lignin react readily with free radicals and cations, further evidence seems required to show that the chemical cross-links between lignin and hemicellulose are not actually artifacts.

PERIODATE OXIDATION

Given a material such as a polysaccharide or a lignin-carbohydrate complex, the problem of determining the nature of the substitution pattern on individual monomeric sugar units can be approached by means of oxidation with periodates (109, 110, 111). This simple reaction is quite specific in that it oxidizes only 1,2-glycols, and it does so quantitatively. However, at high pH, temperature or concentration of periodate, the oxidative selectivity of the reagent is lost, and "over-oxidation" may occur, which leads to erroneous results. Over-oxidation takes place mainly when oxidation of the 1,2-glycol groups in the reducing end-group produces a malonaldehyde derivative possessing an activated hydrogen atom. The main path is then by way of a substituted hydroxymalonaldehyde which in turn is oxidized to a glyoxylyl ester; its hydrolysis exposes the non-reducing unit to complete oxidation (109). However, at or below room temperature, in weakly acid (pH 3.5-5), preferably buffered, solutions, over-consumption of periodate may be diminished, because the intermediary formyl and glyoxylyl esters may fail to hydrolyze, and the reaction can still be used for structural analysis.

Timell was the first to locate the position of acetyl groups in a hemicellulose by means of oxidation with periodate (12). By extracting a white birch holocellulose with dimethyl sulphoxide, he had obtained a xylan containing 9.33% of acetyl groups. The yield of this xylan was 50%. The uronic anhydride, methoxyl and acetyl contents suggested the presence of one 4-O-methyl-D-glucuronic acid residue and 3.6 acetyl groups per 10 xylose units. Simple calculation showed that if the acetylation was random, approximately 6.9 moles of periodate should be consumed for each re-

peating unit of ten xylose and one 4-O-methyl-D-glucuronic acid residues. For only single or double substitution at each xylose residue, the minimum and maximum consumption were shown to be 6.4 and 8.2 moles, respectively. When the isolated acetyl xylan was oxidized with a 0.05 M solution of sodium metaperiodate at room temperature in the dark for various length of time, it was shown to consume 6.6 moles of periodate after extrapolating the almost linear conversion-time curve to zero. This suggested that most xylose units were substituted with only one acetyl group, and the result was later confirmed by Bouveng (84). In a separate experiment, Timell oxidized the acetylated hemicellulose at a lower temperature (+4°) to eliminate possible over-oxidation. After hydrolysis of the oxidized product with acid, the only detectable sugar found in the hydrolyzate was xylose. This was clear evidence that a number of xylose units had originally been protected by acetyl groups at C-2 and C-3. Since no glucuronic acid was found, it must have been unsubstituted.

Later Koshijima (112) and Meier (113) applied periodate oxidation to a glucomannan isolated from pine holocellulose with retention of acetyl groups, and also to a de-acetylated product. The acetylated hemicellulose after oxidation and hydrolysis yielded glucose, mannose, and a small amount of xylose. When the de-acetylated sample was treated in the same way, less than half as much mannose was found, although the quantities of other sugar residues remained constant. The authors considered that these results indicated the acetyl groups were linked to C-2 or C-3 position of the mannose units in the original polysaccharide. Their conclusion has recently been corroborated by Katz (114) in a more detailed study.

OBJECTIVES

The great disadvantage in attempting to use the Björkman's "lignin-carbohydrate complex" for chemical studies is that the preparation of the complex requires a long time. However, the isolation of a "xylan amide" in good yield by extraction with hot water from milled wood previously treated with liquid ammonia has already been described in this thesis. Therefore, it appeared possible that isolation of a fraction similar to the Björkman's complex might be achieved in a much shorter time by applying the same procedure on extractive-free wood. Examination of this complex by electrophoresis and periodate oxidation might be expected to give new information on the nature of the linkages present.

RESULTS AND DISCUSSION

ISOLATION OF STARTING MATERIALS

The effect of duration of milling on the yield of material extractable by hot water was first studied. Samples of extractive-free white birch wood were milled without the addition of liquid for various times at 4°, and then extracted with hot water, with the results shown in Table 9.

The ash content of the milled wood and the Klason lignin content of the "acetyl xylan-lignin complex" (henceforth called LXC) appeared to be independent of the time of milling. This was quite different from Björkman's observations (72) on the wood milled in toluene where the ash content increased with the time of milling while the Klason lignin content of the "lignin-carbohydrate complex" (henceforth called LCC) decreased. Björkman was able to extract an additional quantity of "milled wood lignin" by milling the isolated LCC again. In both types of "complexes" the actual lignin content is probably higher than those estimated by the standard Klason lignin determination, because hardwood is known to contain a significant amount of acid-soluble lignins (72). The sugar contents of LXC and LCC are compared in Table 10. The compositions of both types of "complexes" clearly indicate that the predominate hemicellulose is xylan with lesser amounts of other sugars which were all present in the original wood itself.

After the conditions had been established, LXC was prepared from extractive-free white birch wood in large quantity. A total of 20 g. was isolated. It contained 4.16% of Klason lignin and 14.77% of acetyl groups. Bjorkman's LCC was prepared from white birch in a yield of 15.6% after repeated extraction of the milled wood by dimethyl sulphoxide for 1 year. This LCC contained 11.31% of Klason lignin and 11.55% of acetyl groups.

TABLE 9

Extraction of Milled White Birch Wood with Hot Water

Type of "Complex"	Duration of Milling	% Ash in Milled Wood	% Yield[a] of Extract	% Klason Lignin in Extract
LXC[b]	15 h.	0.51	17.13	6.02
	25 h.	0.50	22.93	5.96
	50 h.	0.50	28.40	5.83
LCC[c]	48 h.	-	15.60	11.31
LCC[d]	48 h.	-	15.00	13.00

(a) Based on oven-dry wood.

(b) "Lignin-xylan complex" isolated by extraction of milled wood with hot water.

(c) "Lignin-carbohydrate complex" isolated by the Björkman procedure from white birch.

(d) Result of Björkman (72) for silver birch wood.

Table 10

Relative Sugar Contents of LXC from White Birch and LCC from Silver Birch

Type of "Complex"	Duration of Milling (hrs.)	% Carbohydrate Content					
		Uronic Acid	Xylose	Galactose	Glucose	Mannose	Arabinose
LXC	15	9.0	72.3	3.8	6.9	6.0	2.0
	25	14.6	63.6	2.9	6.0	8.8	4.1
	50	7.3	82.8	1.0	3.8	4.1	0.9
LCC[a]	48	14.3	73.7	7.7		2.6	1.7

(a) Results of Bjorkman (72) for silver birch, recalculated.

Ammonolysis experiments confirmed the presence of uronic ester groups in both LXC and LCC. The infrared spectra of the "complexes" are completely identical in every respect (Figure 13). The presence of lignin is indicated by the band at 1,500 cm^{-1}. Since these materials contain both acetyl groups and intact uronic ester bonds, yet are much simpler than the original whole wood, they clearly qualify as suitable materials for the study of the points of attachment of the uronic ester bonds.

STUDIES ON URONIC ESTER CROSS-LINKS

AMMONOLYSES OF LXC AND LCC

The Klason lignin content of LCC is almost three times that of LXC. If the hydroxyl groups of lignin are esterified with the 4-O-methylglucuronic acid moiety in xylan, ammonolysis of the uronic ester-lignin cross-links in LCC should afford a product higher in amide nitrogen content than that from LXC. Samples of LXC and LCC were subjected to ammonolysis as previously described. After extraction with methanol to remove acetamide, the samples were dissolved in dimethyl sulphoxide and recovered through precipitation from a mixture of dichloroethane- ethanol (2:1) which has been reported (72) to be effective in purifying polysaccharide material containing entrained lignin. The amide nitrogen content of the ammonolyzed LCC was found to be 0.22%, and that of LXC, 0.21%. These results suggest that the 4-O-methylglucuronic acid in xylan is not esterified with any hydroxyl groups in lignin, and are further supported by the results of alkaline hydrolysis of the "complexes".

ALKALINE HYDROLYSIS OF LCC AND LXC

Samples of LCC and LXC were hydrolyzed in aqueous alkali, and

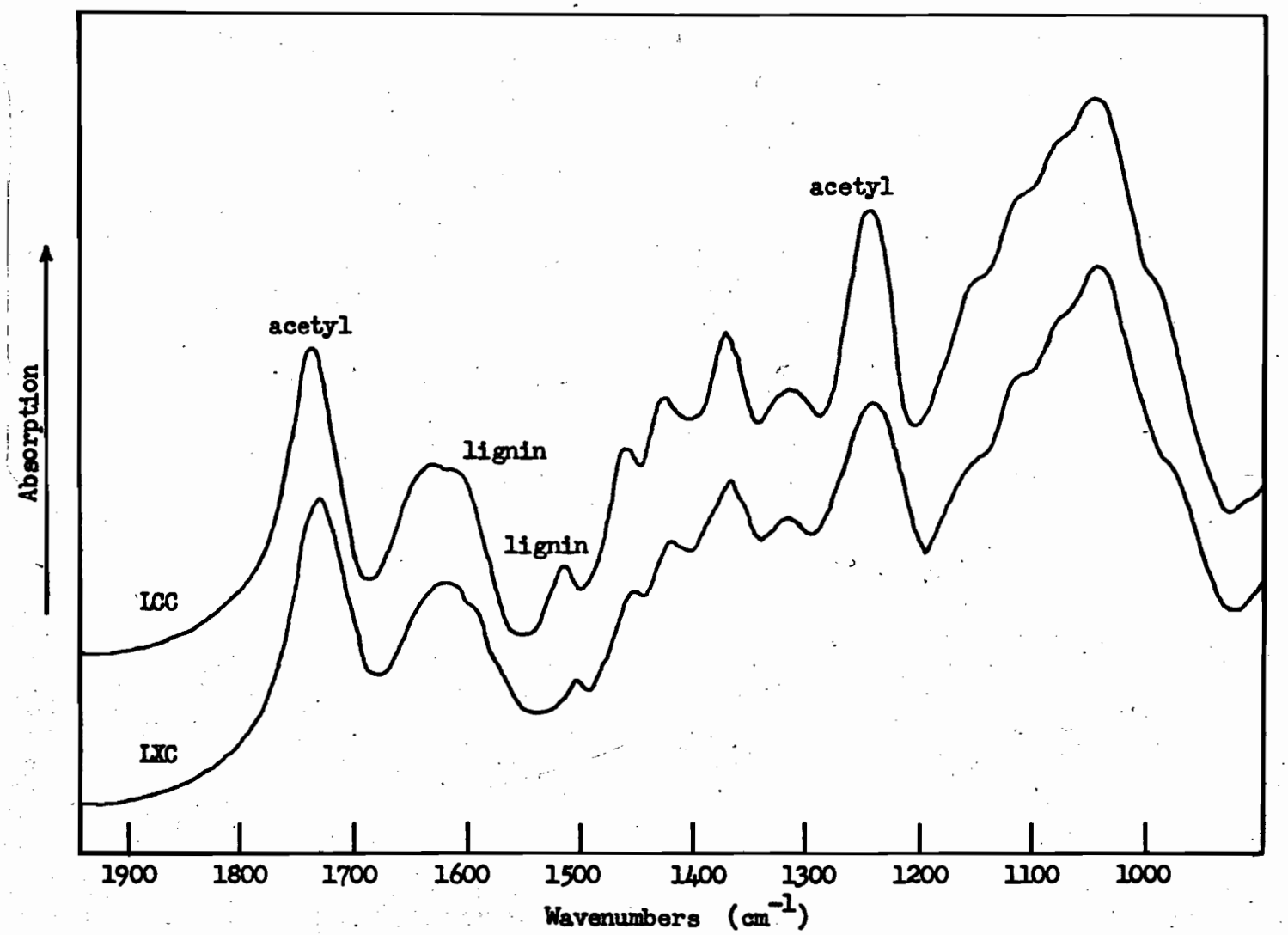


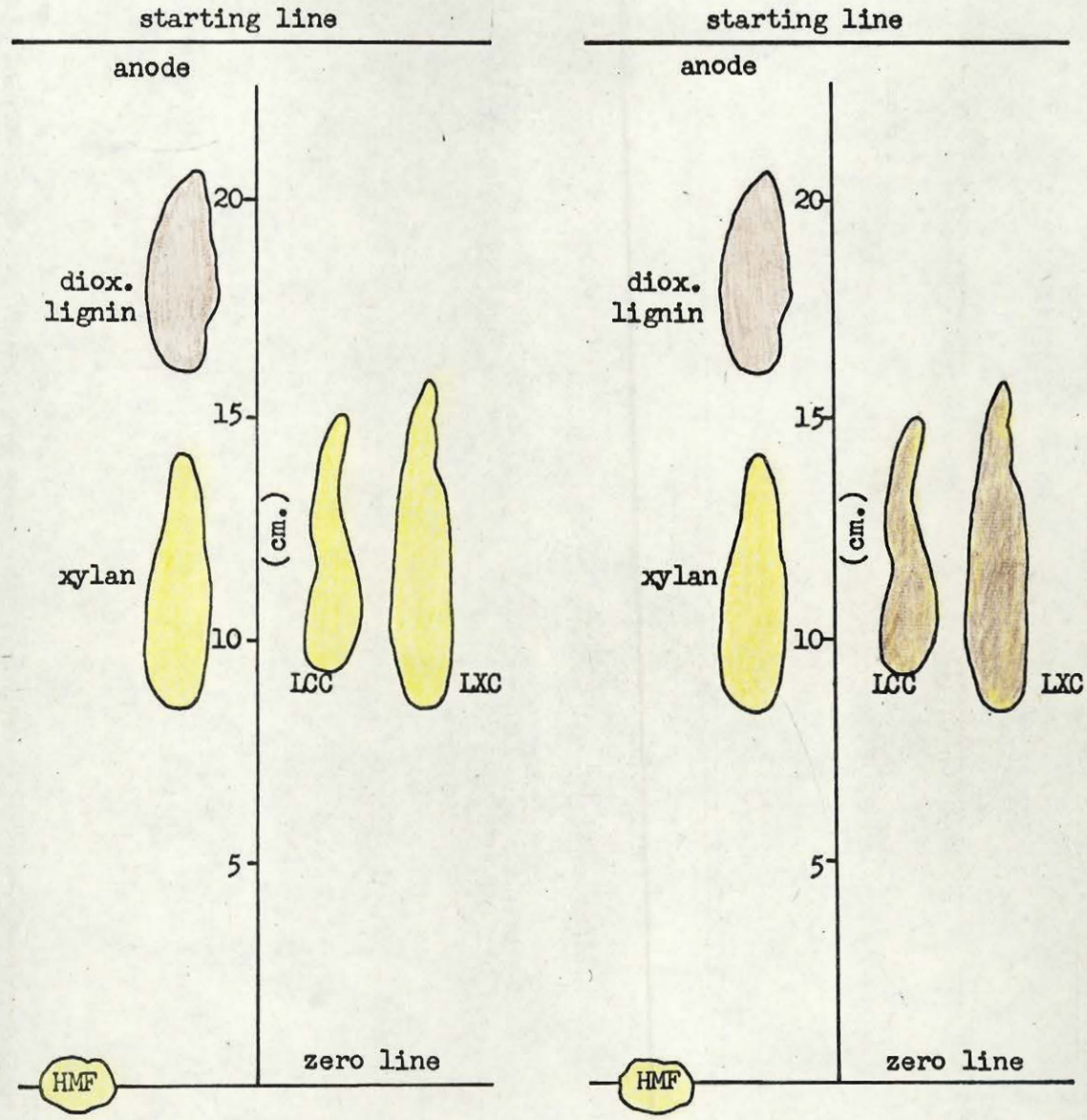
Figure 13 Infrared Spectra of LCC and LXC

"purified" by reprecipitation. That the Klason lignin contents of the saponified samples remained unchanged (Table 11) only indicated the presence of lignin in the samples and provided no useful information on whether the lignin was still in actual chemical combination with the hemicellulsoe. However, the failure to separate lignin from hemicellulose in LCC and LXC by glass-fibre paper electrophoresis lent additional support to the results obtained from ammonolysis and saponification experiments. Therefore, linkages, if any, between lignin and carbohydrate are not likely to involve the uronic ester type, but rather some form of combination which is resistant to the action of alkali.

GLASS-FIBRE PAPER ELECTROPHORESIS OF "LIGNIN-CARBOHYDRATE COMPLEX" AND THE NATURE OF THE LINKAGE

In his study of LCC by electrophoresis, Lindgren (115) neglected to demonstrate whether a simple physical mixture of lignin and polysaccharide could be completely separated. As a preliminary to the present work, this point was checked with a 1:1 mixture of birch dioxane lignin and xylan. Good separation was found. After spraying with p-anisidine and heating, the bright yellow spot with a relative movement of 8-14 cm. corresponded to xylan and the other spot, coloured dark purple, with a relative movement of 16-21 cm. was similar to isolated birch dioxane or milled wood lignin (Table 12).

When the LCC and LXC were run similarly, only single spots appeared on the paper. The spots first gave the bright yellow colour of hemicellulose with p-anisidine after heating for 20 minutes and later, after ten minutes more, part of the spot turned dark purple indicating the presence of lignin (Figure 14).



after heating for 20 min. after heating for 30 min.

(HMF = hydroxymethyl furfural)

Figure 14 Glass-Fibre Paper Electrophoretograms of Lignin and Hemicellulose Materials

Table 11

Klason Lignin Contents of Original and Saponified LCC and LXC

Complex	% Klason Lignin	
	Before Saponification	After Saponification
LCC	11.31	8.56
LXC	4.16	3.97

Table 12

Relative Electrophoretic Movements of Lignin and Hemicellulose Materials on Glass-Fibre Paper Electrophoretograms

Material	Relative Movement[a] in cm. Lignin	Xylan	Complex
Birch Dioxane Lignin	15-19.5	-	-
Birch Milled Wood[b] Lignin	15-19	-	-
Birch Xylan	-	6-15	-
Xylan and Birch Dioxane Lignin Mixture	16-21	8-14	-
LCC	-	-	9-15
LXC	-	-	8.5-16
Acid Hydrolyzed LCC	17-21 (?)[c]	5-13.5	
Acid Hydrolyzed LXC	-	7.5-13.5	

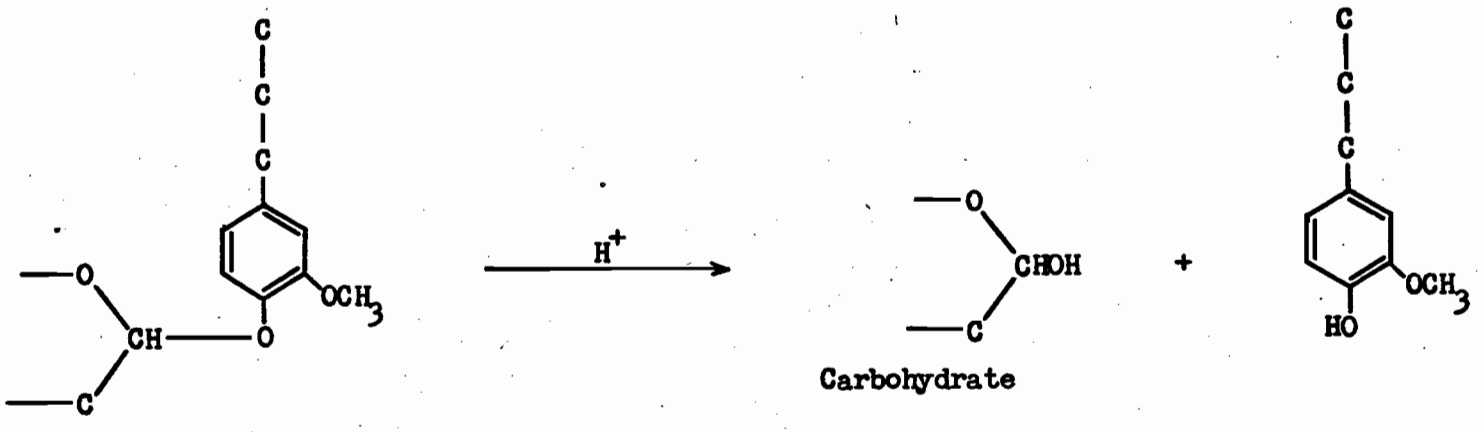
(a) Relative to hydroxymethyl furfural.

(b) Gift of Dr. D.A.I. Goring.

(c) Very faint spot.

Since the LCC and LXC samples were prepared in 0.1 N sodium hydroxide solution and the electrophoresis was also run in an alkaline medium, the failure to separate the lignin from hemicellulose by this method further supported the results obtained from ammonolyses and saponification of these "complexes". If there are any covalent linkages between lignin and hemicellulose in LCC and LXC, they must be alkali-resistant and not likely to be cross-links between uronic ester and lignin.

Several acid-hydrolyzable types of lignin-carbohydrate linkage have been proposed. These are the phenyl glycosidic (22, 28), hemiacetal or acetal (64), and benzyl ether bonds (116) (Figure 15 and 16). To determine whether the LCC and LXC contained any acid-hydrolyzable linkage between lignin and hemicellulose, samples of the "complexes" were hydrolyzed with acid. After the acid was neutralized with sodium bicarbonate, and the solutions were evaporated, the residues were extracted with 17.5% sodium hydroxide and filtered to separate the alkali-insoluble materials. Small samples (35 µl.) of the alkali extracts were resolved by glass-fibre paper electrophoresis which gave only single spots with movement and colour (after spraying with p-anisidine and heating) identical with xylan (Table 12). In the case of acid-hydrolyzed LXC, only 8.5% of the original material was recovered as a light grey precipitate which contained 4.66% of Klason lignin. Because of the small amount of the material recovered, the Klason lignin determination was probably inaccurate and could not be considered as representative. No Klason lignin determination was made on the even smaller quantity of the precipitate recovered from LCC after acid hydrolysis.



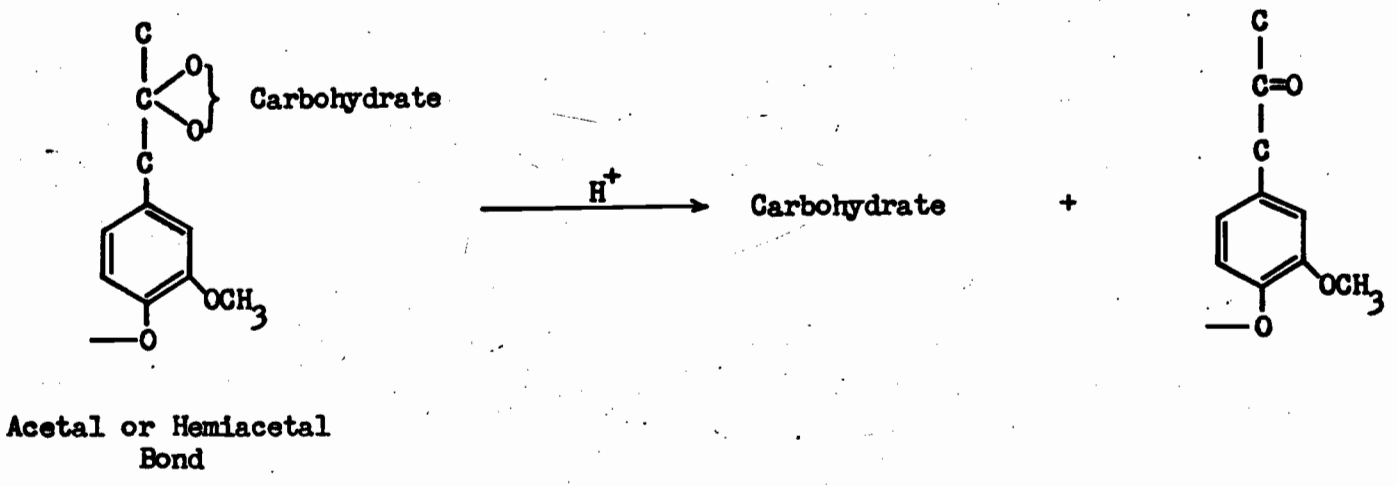


Figure 15 Lignin-Carbohydrate Bonds and Their Hydrolyses

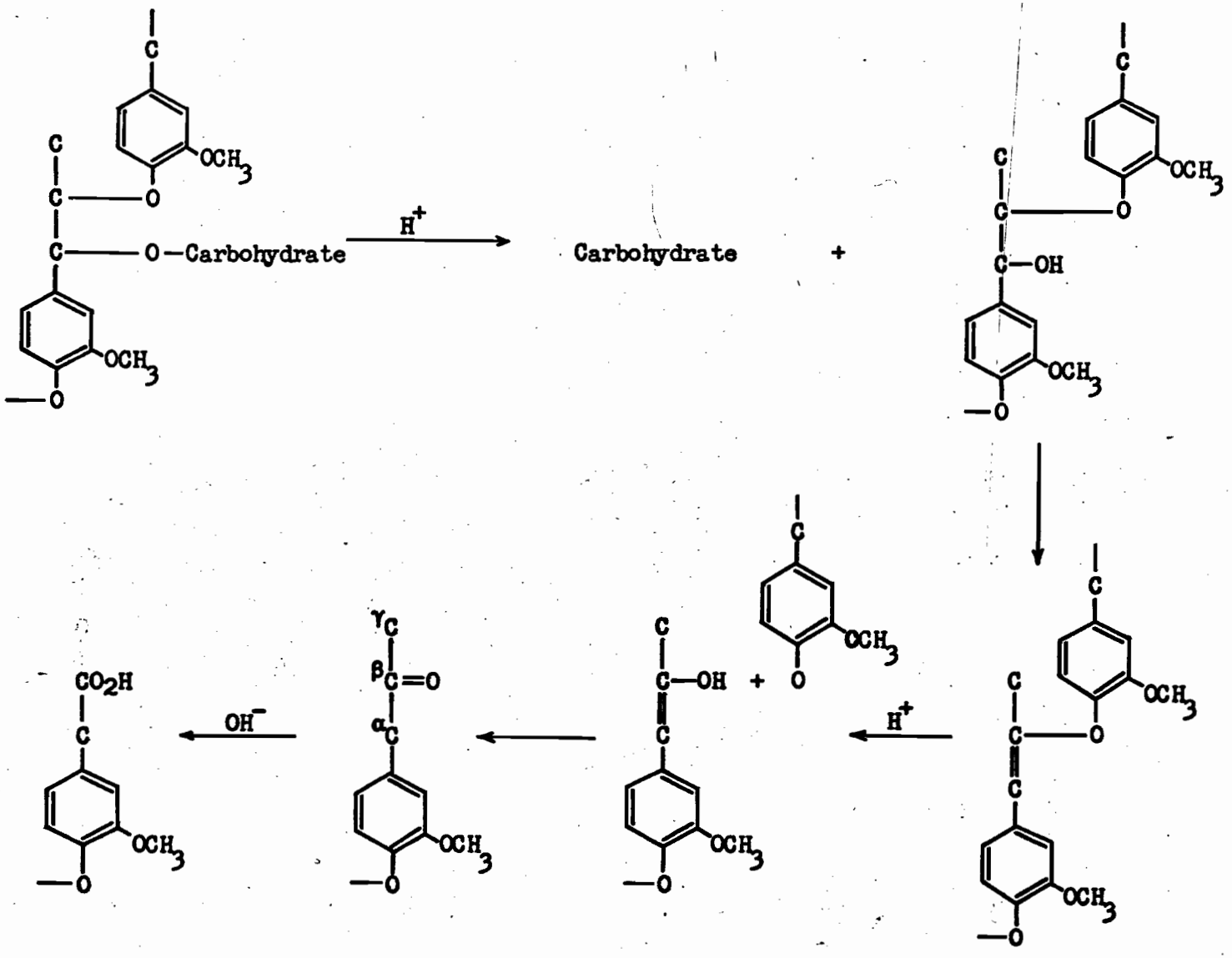


Figure 16 Benzyl Ether Type of Lignin-Carbohydrate Linkage and Its Hydrolysis

The alkali-insoluble residues were examined by infrared spectroscopy. Spectra (Figure 17, I and II) typical of hardwood lignin (64) were obtained which showed no absorption due to free carboxyl or β-keto groups near the 1,730 cm^{-1} region. However, after the alkali-insoluble residues had been thoroughly washed with dilute hydrochloric acid, a new band at 1,720 - 1,730 cm^{-1} appeared (Figure 17, I' and II') suggesting the presence of free carboxyl groups in these lignin samples. The presence of carboxyl groups was interesting, because after the acid hydrolysis of acetal, hemiacetal or benzyl ether type of linkage between lignin and polysaccharide (Figure 15 and 16), β-keto groups should be released. They normally absorb in the 1,710 $\pm$ 15 cm^{-1} region of the infrared spectra. The formation of carboxyl groups was probably the result of alkaline degradation of the β-keto groups (117) or the guaicylglycerol β-coniferyl ether units in lignin (118) formed after the mild acid hydrolysis of the lignin-carbohydrate bond.

PERIODATE OXIDATIONS OF LCC AND LXC

Ammonolysis, saponification and electrophoresis experiments have eliminated the possibility of cross-links between 4-O-methylglucuronic acid and lignin. The remaining alternatives are: (1) intermolecular esterification with other hemicellulose including xylan, (2) intramolecular esterification with hydroxyl groups belonging to the same xylan chain, and (3) lactonization on the C-3 hydroxyl group of the 4-O-methylglucuronic acid itself.

As mentioned previously, Bouveng (84) found that 58.1% of the xylose residues in the xylan of birch were unsubstituted by acetyl groups;

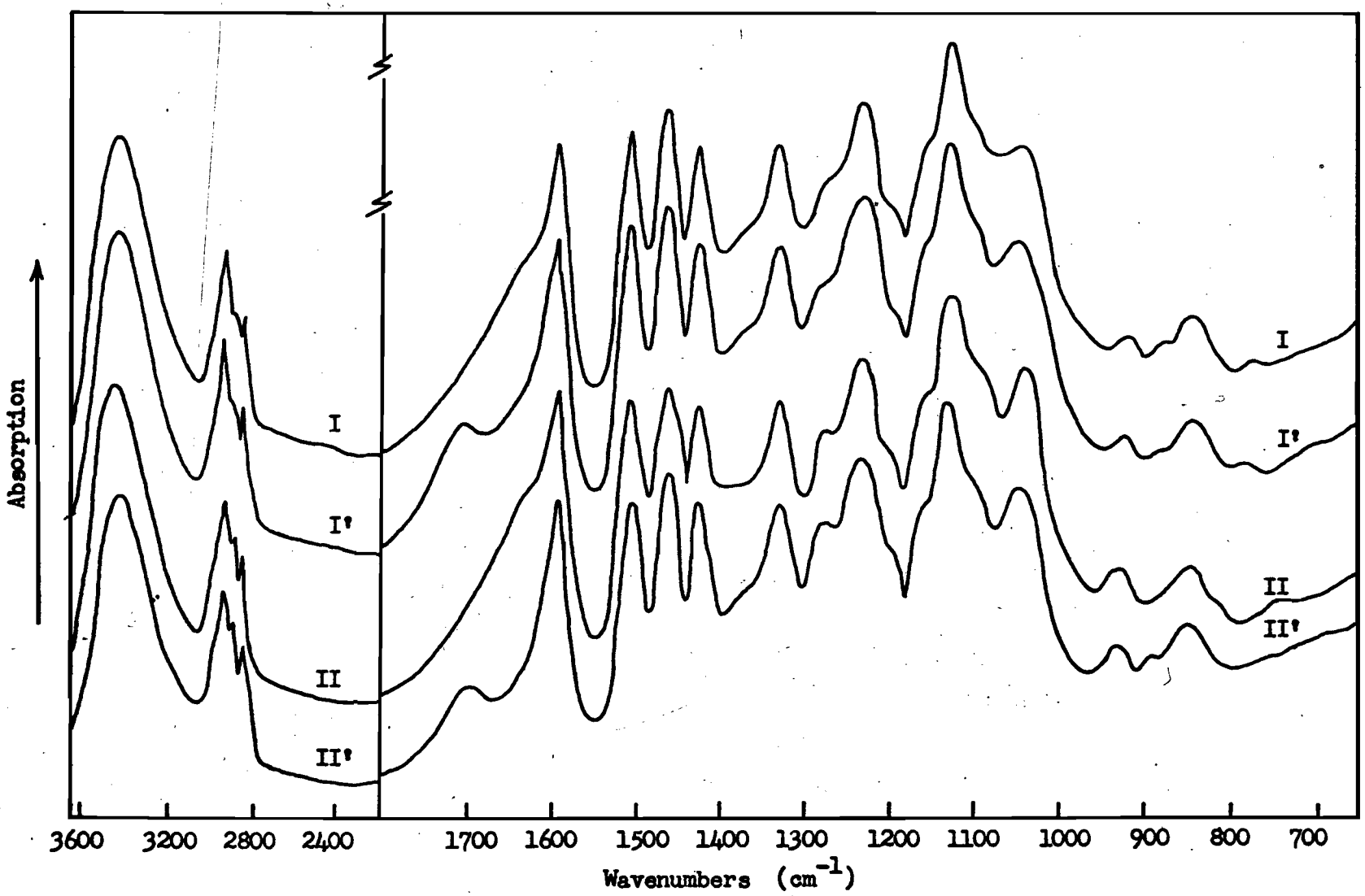


Figure 17 Infrared Spectra of Alkali-Insoluble Residues from Acid-Hydrolyzed LXC (I) and LCC (II), and the Residues after Treatment with Acid (I' and II')

11.8% of the anhydroxylose units were acetylated at the C-2 position, 24.0% at C-3 position and 6.1% at both C-2 and C-3 positions. The larger part of the mannose residues in white birch wood has been found (119) to originate from a glucomannan (about 3% of the wood) containing glucose and mannose in a ratio of 1:1.1 linked by β-(1-4) glycosidic bonds. It has a minimum of 70 hexose residues per average molecule. The small amounts of galactose (0.6% of the wood) and arabinose (0.5% of the wood) in white birch wood are probably constituents of a pectic polysaccharide (82) which has been found in larger quantities (4.6% of the bark) in the inner bark of white birch (120). Gillham and Timell (29) have isolated a galactobiose from a white birch α-cellulose by partial hydrolysis and concluded that the mode of linkage between most galactose units in the wood was β-(1-4) in nature. The linkage between arabinose units is still unknown, but it is likely that they associate with pectic materials as non-reducing side-chains in the furanose form (120).

With an understanding of the structure of the hemicellulose present in white birch wood, periodate oxidation of LCC and LXC will afford valuable information on the origin of the hydroxyl groups esterified with the 4-O-methylglucuronic acid. If the uronic acid is esterified with hydroxyl groups located at C-2 or C-3 of the hemicellulose containing galactose, arabinose, glucose or mannose residues (such as glucomannan) periodate oxidation of the LCC and LXC followed by hydrolysis and paper chromatography should reveal the presence of these sugars. On the other hand, if the uronic acid is lactonized with the hydroxyl group on C-3 of the same monomer unit the presence of 4-O-methylglucuronic acid should be detected in the hydrolyzate. However, after oxidation and hydrolysis, only

D-xylose was actually found by paper chromatography. No traces of aldobiouronic acid or 4-O-methylglucuronic acid were observed.

Thus the periodate oxidation of LCC and LXC eliminated the possibility that the ester bonds represented lactone groups or cross-links with secondary hydroxyl groups in the minor hemicellulose. However, these results alone did not permit a decision as to whether the ester bonds might involve the primary hydroxyl groups at C-6 of glucose and mannose or C-5 of arabinose units. Since about 70% of the uronic acid present in the wood is in the ester form (or 3.22% based on wood), and the minor hemicelluloses (such as glucomannan, galactan etc.) amount to less than 4% of white birch wood (62), it is unlikely that the primary hydroxyl groups of these sugar units can be esterified with uronic acid. A more plausible alternative is that the hydroxyl groups are from the xylan itself. The esterification may be intra- or intermolecular, but only the secondary hydroxyl groups at C-2 and C-3 positions of the xylan can be involved. The information derived from periodate oxidation of LCC and LXC further indicates that if there is a chemical combination between lignin and carbohydrate in these "complexes", the linkage will involve only the C-2 or C-3 position in the xylan. The hydroxyl groups located on C-2 and C-3 of the uronic acid side-chain are completely unsubstituted.

CONCLUSIONS

Based on these results alone, a definite conclusion concerning the nature and the type of lignin-carbohydrate linkage in wood cannot be reached. However, a tentative interpretation in favour of

Freudenberg's theory for the formation of lignin-carbohydrate bond can be considered.

In plant metabolism, according to Freudenberg, Nord, Schubert, and others (116, 121), an acid of the C_6C_3 group, such as phenylpyruvic acid (III in Figure 18) or phenylalanine (IV), is formed from glucose via shikimic acid (I) and prephenic acid (II). Acid-III or -IV is then converted in the cambium (a zone of living cells between bark and wood) into coniferyl alcohol (V) and coniferin (VI). The glucoside-VI diffuses into the freshly grown cells between cambium and mature wood, and is split into coniferyl alcohol and glucose by β-glucosidase which is localized in this region. The coniferyl alcohol is then dehydrogenated by the enzyme laccase (which is only found in the immature cells) into a radical (VIII). A quinone methide (X) is formed by the combination of mesomers (IX) of the radical-VIII. Stabilization of X with hydroxyl groups from polysaccharide (or water) gives the polysaccharide ether of guaiacylglycerol β-coniferyl ether (XI). Further dehydrogenation of XI by laccase results in the formation of lignin containing grafted polysaccharide. When quinone methide (X) was prepared <u>in situ</u>, Freudenberg (123) found that sucrose could be used to stabilize X, and a sucrose ether of XI was actually isolated, which strongly supported the theory of formation of the polysaccharide ether (XI) <u>in vivo</u>.

The lignin-polysaccharide linkage as shown in XI should be resistant to alkali, thus accounting for the failure to separate the small amount of lignin from LCC and LXC by treatment with sodium hydroxide or electrophoresis (run in 0.05 N sodium hydroxide). On the other hand, this benzyl ether linkage should be highly susceptible to hydrolysis by acid

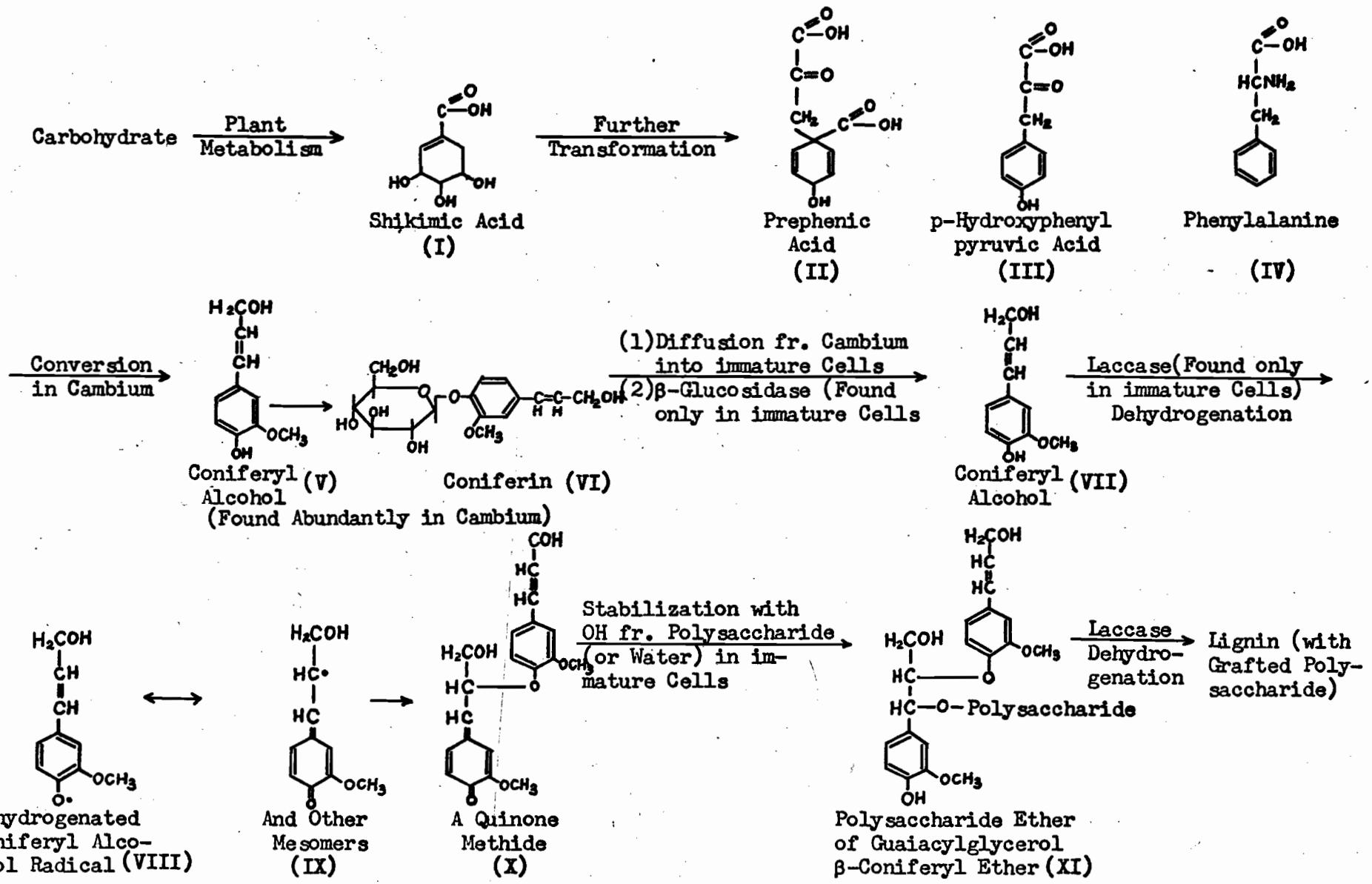


Figure 18 Freudenberg's Theory of Lignin-Carbohydrate Bond Formation

through a S_N1 mechanism. The α-hydroxyl group liberated may further condense with other parts of the lignin molecule (116) which are simultaneously undergoing hydrolysis or condensation in the presence of acid. The small amounts of lignin obtained as alkali-insoluble material after mild acid hydrolyses of LCC and LXC are, therefore, significant.

As mentioned earlier, only xylose was detected after hydrolysis of the periodate oxidized LCC and LXC. This observation suggests that the benzyl ether linkage between lignin and polysaccharide (XI) must involve xylan. The inference is supported by the fact that lignin is formed in the presence of hemicellulose and partially crystalline cellulose (122). In these circumstances, the quinone methide intermediate (X) is most likely to stabilize itself with hydroxyl groups from xylan, especially as the xylan is present as amorphous aggregates on the surface of the crystalline cellulose fibrils and also slightly penetrates into them (122, 123).

Although no unequivocal results have been obtained from the periodate oxidation, saponification, electrophoresis and acid hydrolysis of LCC and LXC, the present research does provide some evidence that whatever linkage does exist between lignin and carbohydrate, it must be stable in alkali, and labile in acid, in agreement with the theory of Freudenberg. It also tends to confirm previous speculation that lignin is covalently attached to xylan.

EXPERIMENTAL

MATERIALS

LCC and LXC were prepared from the same batch of extractive-free white birch wood meal as described in Part II.

ANALYTICAL PROCEDURES

Sugar analyses were performed using the o-aminodiphenyl spectrophotometric method of Timell, Glaudemans and Currie (97).

Ammonium and amide nitrogen contents were determined by the modified method already described.

Ash and Klason lignin contents were determined as described in Part II.

INFRARED SPECTROSCOPY

Infrared spectra were recorded on a Unicam S.P. 100 prism-grating spectrophotometer.

ISOLATION OF LXC

In preliminary experiments designed to study the effect of milling on the yields of hot water extract and their Klason lignin contents, 20 g. samples of the extractive-free white birch wood meal were ground for 15, 25, and 50 hours followed by hot water extraction as described previously in Part II for the isolation of "xylan amide" from liquid-ammonia-treated wood. The result of this study was given in Table 9. For large batch extraction, 80 g. of the milled wood was refluxed for 1 hour with 8 litres of distilled water. The aqueous extract was filtered with-

out cooling, concentrated under reduced pressure at 45° to a convenient volume (200 ml.), dialyzed against distilled water for 3 days, and then again concentrated. Precipitation in ethanol (2 litres) gave 20.1 g. (25.0% based on wood) of grey white material which was later purified according to a modified Björkman's procedure (72) as described below.

PURIFICATION OF LXC

The crude LXC was dissolved in 200 ml. of dimethyl sulphoxide and then ejected from a hyperdemic syringe into 2 litres of a well-stirred mixture of dichloroethane and ethanol (2:1). The precipitate was washed with petroleum ether (b.p. 30-60°) and dried over phosphorus pentoxide; yield: 17.7 g. Klason lignin content of this purified LXC was 4.16%

ISOLATION OF LCC

The LCC was isolated from extractive-free birch wood meal according to Björkman's standard procedure (72). Twelve-gram samples of the wood were suspended in toluene and milled in the ball mill for 48 hours at 4°. The milled wood and toluene were separated from the balls by filtration through a large Büchner funnel. A total of 75 g. of milled wood was collected. The toluene was removed by centrifugation and the wood was suspended in 350 ml. of dioxane containing 4% of water. The dioxane solution was renewed every day for 15 days. After the extraction of "milled wood lignin", the residual wood was dispersed in dimethyl sulphoxide (350 ml.) to extract the LCC. At the end of 5 days, the solvent was separated from the wood by centrifugation, and the extraction was continued with fresh solvent (renewed every 4

months) for 1 year. Approximately 1.5 litres of the dimethyl sulphoxide extract were collected. Crude LCC was obtained in a yield of 22.6% (16.9 g.) by precipitation in 9 litres of ethanol.

PURIFICATION OF LCC

The crude LCC was dissolved in 200 ml. of 50% acetic acid and centrifuged at 1,500 rpm for 2.5 hours. The supernatent liquor was decanted, filtered through a sintered glass funnel (medium porosity), then ejected from a hyperdermic syringe into a vigorously stirred mixture of dichloroethane and ethanol (2:1). After having been separated by centrifugation, the precipitate was dried and reprecipitated in acetone (2 litres) from a solution dimethyl sulphoxide (200 ml.), washed once with acetone, three times with petroleum ether (b.p. 30-60°) and dried in air. Purified LCC was completely soluble in cold water, and gave a dark green solution which was dialyzed against distilled water for 3 days to eliminate low molecular weight materials. The non-dialyzable fraction was recovered by precipitation in ethanol, washed with ether and dried *in vacuo* over phosphorus pentoxide. Yield of the grey white material was 11.7 g. (15.6% based on wood). The Klason lignin content of the purified and dialyzed LCC was 11.31%.

AMMONOLYSES OF LCC AND LXC

Two-gram samples of LCC and LXC were treated with 50 ml. of liquid ammonia under pressure at room temperature as previously described. After evaporation of the ammonia, the samples were extracted with methanol in a Soxhlet extractor to remove acetamide, dried in air and dissolved in

30 ml. of dimethyl sulphoxide. Precipitation in dichloroethane-ethanol mixture (2:1, 300 ml.) gave 1.9 g. of LCC (amide nitrogen: 0.22%) and 1.75 g. of LXC (amide nitrogen: 0.21%).

SAPONIFICATION OF LCC AND LXC

LCC (1.5 g.) was treated with 1 N sodium hydroxide (75 ml.) at room temperature for 48 hours under nitrogen atmosphere. After the alkali solution was neutralized with acetic acid, the LCC was precipitated in ethanol, filtered and dried in air. The saponified LCC was then dissolved in dimethyl sulphoxide (25 ml.), reprecipitated in a mixture of dichloroethane and ethanol (2:1, 300 ml.), filtered, washed with ethanol, ether and then dried in air. Yield of the saponified LCC was 1.20 g. This treatment was claimed (72) to retain the "lignin-like" material in the solution. The Klason lignin content of the saponified LCC was 8.56% (before saponification, 11.31%)

LXC (2.0 g.) after the same treatment (yield: 1.53 g.) had a Klason lignin content of 3.97% (before saponification, 4.16%).

GLASS-FIBRE PAPER ELECTROPHORESIS (115)

Glass-fibre paper (53 x 13 cm., Schleicher and Schull, USA, No. 26) was washed with a phosphate buffer solution (pH 6, 1.6 ml. of 0.1 N NaOH in 20 ml. of 0.1 N NaH_2PO_4), then rinsed thoroughly with distilled water and dried in air. This treatment eliminated preferential adsorption of lignin material on the sheets (115).

The treated glass-fibre paper was dipped into 0.05 N sodium hydroxide solution. Excess electrolyte was removed by blotting with sheets

of heavy filter paper (Whatman 3MM) under pressure for 1 minute, and the glass-fibre paper was placed on top of a horizontal Pyrex glass plate (20.5 x 38 cm.) which had been polished with silicone stopcock grease. Spots of the solutions (35 µl.; concentration ca. 1% for lignins, 10% for LXC, 5% for LCC, isolated xylan, and xylan-dioxane lignin mixture) were applied 1.5 cm. from the edge of the glass plate on the anodic side. A top glass plate (silicone treated) was applied and 25 pounds of weight were placed on it. The ends of the glass-fibre strip were then dipped into the electrolyte (0.05 N NaOH) and the levels of the solution were adjusted to equal distances (3 cm.) from the lower glass plate. The electrolyte solution ascended from both side of the glass-fibre paper and diffused towards the centre; about 30 minutes was required to attain equilibrium. The glass plates were then cooled with salt water (-4°), and 1.8 kilovolts were applied between the graphite electrodes (20.5 x 7.5 cm.) for 45 minutes.

At the end of the run, portions of the glass-fibre paper extending beyond the glass plates were cut off before the weights on top of the glass plates were removed. The electrophoretogram was then dried horizontally with an electric hair dryer, sprayed with a solution of p-anisidine (3 g. p-anisidine in 100 ml. ethanol and 8 ml. concentrated sulphuric acid) and heated for 30 minutes at 110°. Hemicellulose gave a bright yellow spot which appeared after heating for 20 minutes (no change after heating for additional 25 minutes); lignin also gave yellow spot after 10 minutes which soon turned dark purple. LCC and LXC gave bright yellow spots when heated for 20 minutes, but further heating (10 minutes) gave dark purple spots on a diffused yellow background; this indicated

the presence of lignin (Figure 14).

The rate of electro-osmosis was determined by running spots of hydroxymethyl furfural together with the samples. This compound did not move in alkaline or neutral solutions (115), and was strongly coloured by the p-anisidine reagent. The final location of the hydroxymethyl furfural was taken as the zero position, and the movement of the samples (moved against the cathode and electro-osmosis effect) was measured in centimeters between this zero position and the final position of the sample. Lindgren (115) called this distance the "relative mobility" which was rather misleading. In Table 12, this distance is referred to as the movement of the sample relative to hydroxymethyl furfural or "relative movement".

MILD ACID HYDROLYSES OF LCC AND LXC

Two grams of LXC were dissolved in 30 ml. of distilled water and an equal volume of 0.6 N hydrochloric acid was added to give a final acid concentration of 0.3 N. The solution was kept at room temperature overnight, and then refluxed for 1 hour. After neutralization of the acid with sodium bicarbonate, the solution was evaporated to dryness by a stream of air at room temperature. Extraction of the residue with dioxane removed a very small amount (2.1 mg.) of lignin-like material which was not further studied. The residue was then extracted with 17.5% sodium hydroxide (50 ml.) and the extract was filtered to separate the alkali-insoluble material (50 mg.). A small sample (35 µl.) of the alkali-extract was resolved by glass-fibre paper electrophoresis which gave only a single spot with movement and colour (after having been sprayed with

p-anisidine reagent and heated) identical with xylan (Table 12); the remaining alkaline extract was neutralized with acetic acid and poured into large volume (500 ml.) of ethanol. Only 8.5% (0.17 g.) of the original material (2.0 g.) was recovered as a light grey precipitate which contained 4.66% of Klason lignin. Because of low recovery and small amount of the sample, the Klason lignin determination was probably inaccurate and not representative.

LCC after the same treatment gave 68 mg. of alkali-insoluble material. Precipitation of the neutralized alkaline extract in ethanol recovered only 90 mg. of hemicellulose; no Klason lignin content was determined on this sample.

PERIODATE OXIDATION OF LCC AND LXC

LCC (0.5 g.) was dissolved in distilled water (5 ml.) and a sodium metaperiodate solution (20 ml., 0.51 M) was added from a buret to give a solution with final concentration of 0.41 M with respect to periodate (12, 124, 125). The reaction mixture was quickly cooled to 4° and kept at this temperature for 3 weeks in the dark. The change of periodate concentration was followed by the excess arsenite method (126) on 3 ml. aliquotes withdrawn from the reaction mixture at 1 week intervals. No further consumption of periodate was observed after the third week. Excess periodate and iodate ions were removed by precipitation with barium acetate, and centrifugation. The supernatent liquor was decanted and dialyzed against distilled water (2 days), concentrated and hydrolyzed with 1 N sulphuric acid. Paper chromatography (solvent A and B; see Part II) identified the presence of D-xylose only. No trace of any other

sugars or uronic acids could be detected. The small amount of D-xylose (0.12 g.) recovered from the hydrolyzate by precipitation in ethanol had a m.p. and mixed m.p. of 145-146°, $[\alpha]_D$ +18°. Only D-xylose was identified after similar periodate oxidation of LXC.

SUMMARY OF ORIGINAL RESEARCH

1. The presence of uronic acid amide groups in white birch wood treated with liquid ammonia under pressure at room temperature has been confirmed by differential infrared spectroscopy.

2. The amides of xylans have been isolated from liquid-ammonia-treated white birch, aspen, balsam fir, jack pine, and tamarack woods by milling and extraction with hot water, thus demonstrating that a significant proportion of the uronic acid groups in all these species are esterified in their native state.

3. The hydroxyl groups esterified with 4-O-methylglucuronic acid in white birch wood have been located on the C-2 and C-3 positions of xylose units in xylan and were shown not to be on lignin or other carbohydrate constituents of the wood.

4. A "lignin-carbohydrate complex" has been isolated from milled white birch wood by extraction with hot water in a much shorter time than the conventional Björkman method.

5. By application of zone electrophoresis, the linkage between lignin and carbohydrate has been shown to be alkali-resistant but easily hydrolyzed by acid, thus lending support to current theories of the nature of the bond between lignin and carbohydrate. Periodate oxidation of the "lignin-carbohydrate complex" showed that xylose units were probably involved in the cross-linking.

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APPENDIX

EXTRACTION OF WOODS WITH ANHYDROUS LIQUID AMMONIA FOLLOWED BY DIMETHYL SULPHOXIDE

INTRODUCTION

Dimethyl sulphoxide (abbreviated henceforth to DMSO) has been known for over a century (1), but has only recently been made on an industrial scale. It is now becoming increasingly important as an intermediate in synthetic processes, and its solvent properties have been demonstrated for a number of gases, liquids, certain salts and a variety of polymers (1).

DMSO is a colourless, water-soluble, highly hygroscopic liquid that is generally inert, but decomposes violently when in contact with a number of acyl halides and related compounds.

Micheal and co-workers (2) have recently reported the use of DMSO as a solvent in the acid-catalyzed synthesis of water-soluble polysaccharides from simple sugars (D-glucose, D-mannose, D-galactose, and D-xylose) and disaccharides (maltose, cellubiose, and lactose) Removal of the water liberated during the condensation reaction was shown to increase the yields of the synthetic polysaccharide. Some units of the hexoses suffered configurational changes from the pyranose to the furanose form during condensation; poly-D-xylose did not seem to undergo such mutation. All of the synthetic polyoses prepared in this way could be completely acetylated, but attempts at methylation was difficult, perhaps because of considerable branching.

Manley (3) has reported the high solubility of non-ionic cellulose ethers in DMSO, and found it to be the only organic solvent capable of dissolving water-soluble ethyl hydroxylethyl cellulose. Everett and Foster (4) have presented evidence that by fractional precipitation from the DMSO solution, potato amylose could be freed from amylo-

pectin contaminants to an extent that meaningful physical measurements were possible; the results showed that the polymer appeared in a coiled configuration. Killion and Foster (5) have isolated amylose with minimal degradation by dispersing undried potato starch in DMSO and precipitating the amylose by adding butanol.

DMSO has also been used as a solvent in the potentiometric titration of lignin and its model compounds, with 0.05 N potassium methoxide in benzene as the titrant (6). An alkali lignin obtained from black liquor was shown in this way to contain 0.95 milliequivalents of carboxyl groups, and 6.12 milliequivalents of hydroxyl groups per gram.

A systematic evaluation of organic liquids as solvents for isolated lignin was recently reported by Schuerch (7). The liquids were classified in terms of their Hildebrand solubility parameter, k, calculated from the equation:

$$k = \left(\frac{H - RT}{V}\right)^{1/2}$$

where H = molar heat of vaporization (cal/mole)
T = boiling point of liquid in °K
V = molar volume of the liquid, ml.
R = 1.98 cal/mole /°K

Schuerch found that liquids with solubility parameters of 11 or more tend to be relatively good solvents for lignin, while those with parameters much below 11 are poor ones. Thus DMSO, with k = 13.4, is comparable to ethylene glycol (k = 14.2) as a solvent for lignin, and better than ethylene chlorohydrin (k = 11.6) and dimethyl formamide (k = 9.9).

Before the work of Schuerch, the solvent properties of DMSO had

already been explored in delignification studies. Giertz (8) attempted the delignification of spruce chips with pure DMSO as well as with DMSO containing traces of sulphur dioxide, sodium bisulphite, sulphur, phenol or hydrochloric acid at elevated temperatures, and reported that complete separation of fibres occurred with the concurrent removal of large amounts of lignin; carbohydrate-free lignin fractions were recovered by precipitation in water. Similar studies were simultaneously and subsequently carried out in Canada and France. Clermont and Bender (10) have reported that at high temperatures, solutions of sulphur dioxide, nitric oxide or chlorine in DMSO with 5% of water showed excellent pulping properties towards black spruce or aspen sawdust and chips. The resulting pulps had low lignin and high α-cellulose contents. Addition of water to the spent cooking liquor precipitated the lignin. Hossain (9) has shown that at elevated temperatures, DMSO containing 0.2% sulphuric acid had a powerful delignifying action on high-yield spruce sulphite pulps which contained most of the original lignin in a partially sulphonated form and also on spruce wood chips; no delignification was observed when the samples were cooked with pure DMSO. Robert and Doucet (11) contradicted the previous claim of Hossain (9) that no extraction of lignin or polysaccharide occurred in cooking with pure anhydrous DMSO at normal or elevated temperatures. Robert and Doucet extracted 46.6% of the lignin and 53.0% of the pentosan in birch wood by cooking in DMSO for 4 hours at 185°; 14.8% of the lignin and 12.2% of the pentosan were extracted in 4 hours at room temperature. However, Robert and Doucet agreed with Hossain that the presence of a trace of hydrogen ion enhanced the delignification. They also showed that

whether trace amounts of hydrogen ion (ca. 0.2% HCl) were present or not, lignin and polysaccharides were extracted in approximately equal amounts; to obtain a less degraded lignin, the prolonged action of DMSO on wood meal at normal temperature was recommended.

In a study on finely divided wood, Björkman (13) found that after a large proportion of the "milled-wood lignin" was first extracted by dioxane, a grey-white material containing both lignin and polysaccharide could be extracted by DMSO. Later, McPherson (14) purified this "lignin-carbohydrate complex" and chromatographed the material on a Celite column, and obtained a number of fractions differing widely in their lignin contents.

The use of DMSO to extract hemicellulose from hard- and softwood chlorite holocelluloses was first achieved in 1956 by Hägglund and co-workers (12). The extracted hemicellulose still contained a high percentage of the acetyl groups originally present in the wood; more hemicellulose could be extracted from the swollen residue by subsequent treatment with water. Hagglund also mentioned the usefulness of DMSO as a rather selective precipitating agent for mannans. Koshijima (15) extracted pine holocellulose with DMSO plus acetic and boric acid mixtures, and isolated hemicellulose containing considerable amounts of mannose and acetyl groups. Timell (16) tentatively established the structure of a water-soluble acetyl-4-O-methylglucurono-xyloglycan extracted by DMSO from a birch chlorine holocellulose in 50% yield of the total xylan content of the wood. The number- and weight-average D.P. of the polysaccharide as determined by osmometry and light scattering were 180 and 470 respectively.

Previous attempts (17) to extract a hemicellulose by hot water from sugar maple wood treated with liquid ammonia have ended in failure. Yet the mild action and versatile solvent properties of DMSO as reported by other workers suggestthat it might be able to extract a xylan suspected to contain amide nitrogen from such a residual wood (18). The present work indicated that DMSO alone was no better than water in its solvent power when used on woods previously treated with liquid ammonia.

RESULTS AND DISCUSSION

EXTRACTION OF WOODS WITH ANHYDROUS LIQUID AMMONIA

Extraction for 5 hours at room temperature evaluated as optimal by Yan (18) was adopted. Table 1 summarizes the yields of the liquid ammonia extract found by Yan and others together with those obtained in the present experiments. The results show reasonable agreement when the extraction conditions were closely similar.

EXTRACTIONS OF LIQUID-AMMONIA-TREATED WOOD MEALS WITH PURE DMSO OR DISTILLED WATER

The extractions were carried out at room temperature for 42 hours under dry nitrogen on a mechanical shaker; the yields based on oven-dry liquid-ammonia-extracted wood are listed in Table 2. The results presented for water extractions include small quantities of ammonium acetate resulting from the addition of acetic acid to adjust the pH, therefore, the actual yields were a little less than shown. The aqueous extracts were still found to be neutral after 42 hours of extraction. Brown-coloured flakes recovered from the evaporation of DMSO extracts appeared darker than those recovered when water was used as the extracting solvent. In all cases, the wood meals showed considerable swelling in DMSO in less than 15 minutes after contact, and became much darker and semi-transparent. Yields of materials extracted by aqueous or organic solvents from other samples of woods treated with liquid ammonia are summarized in Table 3 for reference.

Table 2 shows that the pretreatment with liquid ammonia did little, if anything, to enhance the solubility of any wood components in

Table 1

Extraction of Woods with Liquid Ammonia at Room Temperature

Investigator	Material	Reaction Time (hrs.)	% Yield (oven-dry basis)	Reference
Yan and Neubauer	Sugar Maple	5	5.69 (large scale)	(17, 18)
	Rye Straw	65	11.4 (small scale)	
	Birch	3 (100°)	8.30 (small scale)	
	Spruce	16	3.00 (small scale)	
Jablonski	Spruce Bark	16 to overnight	6.23-6.75 (small scale) 3.34-3.94 (large scale)	(19)
Milford and Watts	Spruce Holocell.	overnight	6.23 (small scale)	(20, 21)
Milks	Aspen Holocell.	16	10.2 (large scale exhaustive extn.	(22)
Present	Red Maple	5	5.99 (small scale)	
	Birch	5	4.26 (small scale)	
	Aspen	5	1.19 (small scale)	
	Spruce	5	1.12 (small scale)	

Table 2

Yield of DMSO Extract from Woods

Wood	Liq.-Ammonia-Treated Wood		% DMSO Extract fr. Untreated Wood
	% Water Extract	% DMSO Extract	
Red Maple	1.51	1.15	1.13
Birch	1.00	3.50	1.38
Aspen	1.76	0.94	-
Spruce	1.61	1.46	1.35

Table 3

Extraction of Liquid-Ammonia-Treated Woods

Investigator	Material	Total Extn. Time	Vol. of Extracting Liquor	Extn. Temp. °C	pH	Extn. Cycle	% Total Yield	Reference
Neubauer	Sugar Maple	-	EtOH in Soxhlet	-	-	-	0.8	(18)
		6 hrs.	500 g. with 3 l. water	95-97°	-	1 cy/2 hrs.	2.14	
Sanderson	White Spruce Bark	2 days then 3 days	wt:vol=1:10	20° 70°	5	1 cy/day	12.7	(23)
		5 days then 8 days	same	same	5	same	26.0	
		same	same	same	11	same	25.0	
Milks	Aspen Holocell.	10 hrs. then 10 hrs.	616 g. with 5 l. water	25° 70°	5-6	1 cy/5 hrs.	11.35	(22)
Milford and Watts	Black Spruce Holocell.	-	EtOH in Soxhlet	-	-	-	0.65	(20, 21)
		8 hrs. then 8 hrs.	219 g. with 5 l. water	25° 70°	6	1 cy/4 hrs.	19.3	

DMSO compared to distilled water at pH 7. These results appeared to confirm the view of Nelson and Schuerch (24), based on kinetic studies, that the extraction of polysaccharides from wood meal with swelling solvents such as 1-24% sodium hydroxide was a physical process of leaching through fibre structures pre-swollen to an equilibrium condition depending on temperature, concentration of extractant, morphological structure, and amount of lignin present. Even at maximum swelling, the polysaccharides had to diffuse through a stiff microcrystalline gel; thus diffusion occurred at a scarcely measurable rate. If swelling was the prevailing factor inhibiting the "hemicellulose amide" from being extracted by DMSO, then it would be necessary to mill the liquid-ammonia-treated wood into a powdery state (13), even at the risk of slight degradation, to render these polysaccharides extractable by DMSO. However, another possibility could not be overruled. The excessive drying of the wood meals after liquid ammonia extractions might have altered the texture of the wood, and occluded components otherwise easily extractable by DMSO.

The slight pulping action of pure DMSO, which resulted in almost equal amounts of lignin and polysaccharides being extracted, was reported recently (11). The arithmetic difference between Column-3 and -4 of Table 2, them, should give the actual amount of hemicellulose solubilized in DMSO as a result of ammonolysis. In the case of liquid-ammonia-treated birch, 1.38% (second row, Table 2) of the 3.05% extracted by DMSO may have been due to pulping action, and the rest due to liquid ammonia treatment. This remainder amounted to only 2.12% and could not be regarded as representative of the native hemicellulose in wood.

EFFECT OF TEMPERATURE, AQUEOUS DILUTION, AND WOOD TO DMSO RATIO ON THE YIELD OF EXTRACT

Variations in experimental conditions did not give a substantial increase in the yield of extract from liquid-ammonia-treated red maple. An increase in extraction temperature form 25° to 80° gave a slightly higher yield of DMSO extract from red maple as shown in Table 4. There was no convincing reason that this small increase was not solely due to the pulping action of DMSO on wood (11), an action which was irrelevant to this investigation. Extraction near the freezing point of DMSO gave a slightly lower yield than a extraction at room temperature.

The increase in the ratio of DMSO to wood (v:wt.) did not result in higher yield. The 1:15 ratio (wood:DMSO) used was sufficient to give consistent results as shown in Table 5. A lower ratio than 1:15 was found to be inconvenient, because the high degree of swelling of the wood made the extracting liquor barely suffice to cover the meal in a 50-ml. round-bottom flask. At a 1:15 ratio, the meal absorbed the DMSO completely in less than 5 minutes, and gave a dark brown and highly swollen slurry.

Dilution of DMSO with water was recently shown to increase the dissolution of certain xylans (25). The percentage amounts of liquid-ammonia-treated red maple extracted with DMSO at different aqueous dilutions are shown in Table 6. The pH of the extracting liquors (except 100% DMSO) were adjusted by acetic acid as usual, and the yields listed thus included small amounts of ammonium acetate. Table 6 shows that the aqueous dilutions did not increase the yield to any encouraging extent. Only one parameter was changed at a time while all others were held constant in these series of extractions in order to make comparison mean-

Table 4

Effect of Temperature on Yield of DMSO Extract from Red Maple Treated with Liquid Ammonia

Temperature °C	Extract %
19	0.92
25	1.15
80	1.78

Table 5

Effect of Wood to DMSO Ratio on Yield of [illegible] from Red Maple Treated with Liquid Ammonia

Ratio (wt:vol)	Extract %
1:15	1.08
1:30	1.02
1:50	1.02
1:100	1.06

Table 6

Effect of Aqueous Dilutions on Yield of Extract from Red Maple Treated with Liquid Ammonia

% Water in DMSO	% Extract
0	1.15
10	0.97
20	1.06
40	1.17
60	0.97
100	1.51

ingful. A high heat of dilution was noticed upon adding distilled water or wood meals to DMSO; in large scale extractions, this unnecessary warming must be eliminated by pre-cooling the DMSO.

The residual ammonia in the wood meal, which could not be eliminated completely even after prolonged evacuation over phosphorus pentoxide, merited attention because of its possible effect on extraction.

THE EXTRACTION OF BIRCH WOOD MEAL

Since birch meal treated with liquid ammonia gave the most outstanding results in DMSO extractions among the woods so far dealt with, it seemed to merit special attention. DMSO extracted 3.5% of the liquid-ammonia-treated birch at 25°, and 3.75% at 80°. Recalling the similar situation for red maple, the slightly higher yield at 80° also included the possibility of the DMSO pulping action on wood. Therefore, extractions at the higher temperature did not warrant the risk of possible condensation or degradation.

Parallel extractions of liquid-ammonia-treated and untreated birch wood meal showed that within a period of 42 hours at 80°, the amount of hemicellulose (possibly together with ligneous materials) made soluble in DMSO due to the liquid ammonia pre-treatment could be completely extracted. Table 7 shows the yields of several successive extractions with DMSO. Starting from the second extraction, both samples gave similar results which could best be interpreted as only the action of DMSO on wood; the pre-treatment with liquid ammonia had little effect.

Table 7

Successive DMSO Extractions of Birch Wood Meal

Sequence of Extractions	Duration (hrs.)	Untreated Meal (%)	Liq. Ammonia Treated Meal (%)
first	42	1.38	3.75
second	42	0.43	0.49
third	53	0.42	0.55
fourth	84	0.40	0.48
total	221	2.63	5.27

EXPERIMENTAL

PREPARATION OF EXTRACTIVE-FREE WOOD MEALS

Samples of approximately 40 to 60 mesh size birch, spruce, aspen, and red maple meals with unknown history and background were first extracted separately in a Soxhlet apparatus of 200 g. capacity. Extraction was for 36 hours at two cycles of siphoning per hour with a 1:2 azeotropic solution of ethanol and benzene to remove extraneous materials. The greater part of the benzene left in the meal was then removed by soaking the bag containg the meal in ethanol for one hour, then squeezing out the ethanol with the entrained benzene. This treatment was followed by hot ethanol extraction for 24 hours (2 cycles of siphoning per hour). The bag of meal was then removed from the extractor, and hot (60°) tap water was run through the meal for 48 hours. After a 4-hour soaking in distilled water, the meal was air-dried for 3 days, re-screened to separate the 40 to 60 mesh size portion and stored in screw-capped amber bottles so that the moisture content would remain roughly constant over long periods

MOISTURE CONTENT OF THE EXTRACTIVE-FREE WOOD MEALS

One-half to two gram samples were kept in an oven at 110° for 16 hours and the loss of weight was determined. The weighings were done in glass-stoppered weighing bottles on an analytical balance. All determinations were done in duplicate.

TREATMENT OF WOOD MEALS WITH ANHYDROUS LIQUID AMMONIA

Seventeen-gram batches of the wood meals were treated with commercial liquid ammonia. The detailed procedure for treatment and the

construction of the bomb have been described by Yan (18). Throughout the experiment, the access of atmospheric moisture was carefully prevented. All treatments were conducted with a ratio of wood to liquid ammonia of 1:15 (wt:vol) at room temperature for 5 hours. The filtration apparatus suggested by Yan was found to be unsatisfactory, since it retained large quantities of wood meal in its connecting tubes; the Jablonski modification (19) of the apparatus was much more convenient and efficient for this purpose. In the present experiments, their mercury safety valve for liquid ammonia was replace by sodium hydroxide (pellets) drying towers open to atmospheric pressure, because the former might cause unnecessary hazard (26).

EXTRACTION OF LIQUID-AMMONIA-TREATED WOOD MEALS WITH DMSO OR WATER

Duplicate, 1.0 to 3.0 g. samples of liquid-ammonia-treated wood meals, previously dried over P_2O_5 *in vacuo*, were placed in 50-ml. round bottom flasks, and the extracting solvent, DMSO or water, was added to make a wood:liquor ratio of 1:15 (wt:vol). Aqueous suspensions, but not DMSO, were then adjusted to pH 7 by the addition of 10% acetic acid. The flasks were flushed with dry nitrogen, sealed tightly, and shaken mechanically for 42 hours or more at the required temperature. The extract was removed by filtration on a sintered glass filter, and the residual solid was washed with same volume of the extracting solvent. On some occasions, the undried residue was again quantitatively extracted under the same conditions. The extract was centrifuged at 2,200 rpm for 30 minutes to remove colloidal wood particles, evaporated under reduced pressure at 50°, and then dried *in vacuo* over P_2O_5 to constant weight to determine the yield. All yields reported were based on the wood after extraction with liquid ammonia unless otherwise stated.

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