[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Lipids of Wheat Flour. I. Characterization of Galactosylglycerol Components[1]

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A benzene extract of bleached wheat flour was fractionated by Craig distribution between *n*-heptane and 95% methanol into triglyceride, steroid, lipoprotein and lipocarbohydrate fractions. The lipocarbohydrate was further fractionated from acetone into a more soluble and a less soluble component. In a study of these two materials the carbohydrate moiety of the more soluble fraction was identified as β-D-galactopyranosyl-1-glycerol and the carbohydrate moiety of the less soluble fraction as α-D-galactopyranosyl-1,6-β-D-galactopyranosyl-1-glycerol.

A study of the benzene-extractable lipids of bleached wheat flour has demonstrated the presence of at least two galactosylglyceride compounds. The purpose of this paper is to report the characterization of the carbohydrate moiety of these lipids.

In an initial study the benzene extract was separated by counter-current distribution between *n*-heptane and 95% methanol into four distinct fractions. The four fractions consisted of a steroid which precipitated spontaneously from the *n*-heptane, a lipoprotein which was precipitated from both phases with acetone, a triglyceride fraction from the *n*-heptane phase and a fraction containing 20% carbohydrate from the methanol phase. The only material to be described in this paper is the lipocarbohydrate fraction, the remaining materials will be the subject of future studies.

The lipocarbohydrate fraction was soluble in warm acetone but on cooling could be separated into acetone-insoluble and soluble materials, designated fractions I and II, respectively. To date, attempts to obtain completely homogeneous substances by this procedure have been unsuccessful. Analytical data on these two fractions are presented in Table I.

TABLE I

LIPOCARBOHYDRATE FRACTIONS I AND II

	Carbohydrate, %	Fatty acid, %	Nitrogen, %	Phosphorus, %	Chlorine, %
I	29	58	0.59	0.26	4.6
II	12	56	0.18	0.5	3.6

Fractions I and II were subjected to alkaline hydrolysis. The hydrolysates were acidified and fatty acids were extracted with petroleum ether. The aqueous solutions containing the carbohydrate moieties were deionized and finally chromatographed on carbon—Celite columns. Elution with dilute aqueous ethanol yielded two distinct carbohydrate fractions, A and B. Fraction I yielded 10% of A and 90% of B, whereas from fraction II, A and B were obtained in approximately equal quantities. Both A and B were chromatographically homogeneous in three solvent systems and were non-reducing before acid hydrolysis. Upon acid hydrolysis A and B yielded only galactose and glycerol as determined by paper chromatography. Crystallization of fraction A was accomplished from absolute methanol. Fraction B was crystallized from an ethanol—water mixture. Data shown in Table II indicate that fraction A is a monogalactosylglycerol and fraction B a digalactosylglycerol.

TABLE II

ANALYSES OF COMPOUNDS A AND B

	Monogalactosylglycerol Fraction A	Monogalactosylglycerol Theoretical	Digalactosylglycerol Fraction B	Digalactosylglycerol Theoretical
Galactose, %	71	70.9	84	86.4
Glycerol, %	35.3	35.8	22.8	22.9
M.p., °C.	139–140	...	182–184	...
$[\alpha]^{27}D$	+3.77°	...	+86.4°	...

Additional evidence for the presence of galactose as the only carbohydrate component of A and B was obtained by preparing the α-methylphenylhydrazone of galactose from acid hydrolysates of these substances. The over-all yield of this derivative from A and B was 85 and 90%, respectively.

To further elucidate the structure of these compounds, a periodate oxidation study was made. The monogalactosylglycerol (A) consumed 3 moles of periodate and produced 1 mole of formic acid and 1 mole of formaldehyde. These data are consistent only with a galactopyranosyl-1-glycerol structure. The digalactosylglycerol (B) consumed 5 moles of periodate, yielding 2 moles of formic acid and 1 mole of formaldehyde. Thus a galactopyranosyl-1,6-galactopyranosyl-1-glycerol structure was indicated.

Studies of the enzymatic hydrolysis of A and B with specific α- and β-galactosidases confirmed the periodate results. When the di-galactosylglycerol (B) was incubated with yeast α-galactosidase, one-half of the potential reducing power was liberated. Chromatography of the hydrolysis products on a carbon—Celite column resulted in the isolation of galactose and a monogalactosylglycerol which was identical with A. Treatment of either preparation of monogalactosylglycerol with α-galactosidase resulted in no demonstrable hydrolysis. An *E. coli* β-galactosidase preparation gave no detectable hydrolysis of the digalactosylglycerol but cleaved the monogalactosylglycerol at a moderate rate. These results establish a β-linkage of galactose to glycerol in both compounds and an α-linkage of the second galactose residue in the digalactosylglycerol.

The specific rotations of A and B supply supplementary evidence for the nature of the glycosidic linkages. β-Galactosides usually have a very low

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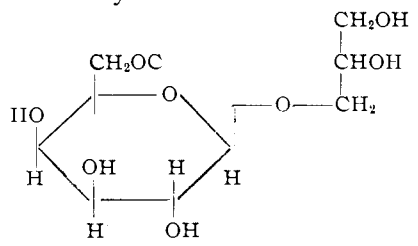
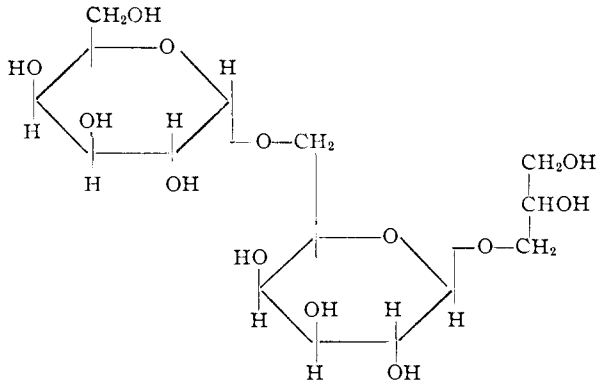
specific rotation (β-methylgalactoside, 0.0[3]) whereas most α-galactosides have specific rotations[4] of 125 to 200°. Thus the low rotation (+3.77°) of the monogalactosylglycerol is characteristic of a β-galactoside while the value of +86.4° for the digalactosylglycerol is about that to be expected from a compound containing one α- and one β-galactosidic linkage.

The following structures are therefore assigned to the two carbohydrates.



β-D-galactopyranosyl-1-glycerol (A)



α-D-galactopyranosyl-1,6-β-D-galactopyranosyl-1-glycerol (B)

Putman and Hassid[5] have reported the isolation of α-D-galactopyranosyl-2-glycerol from the red marine alga *Irideae laminarioides*. Dr. Hassid kindly provided us with a small amount of this material for comparative studies. The alga product was found to be distinct from our monogalactosylglycerol by paper chromatography, optical rotation, melting point, and behavior toward enzymatic hydrolysis. The isolation of two different monogalactosylglycerols from plant sources provides an interesting problem in biochemical relationships.

Experimental

Fractionation of Wheat Flour Lipids.—In the first fractionation a nine transfer, double withdrawal Craig distribution was carried out in the usual manner. The upper phase consisted of four 500-ml. portions of *n*-heptane which had been pre-equilibrated with 95% methanol; the lower phase of five 500-ml. portions of 95% methanol pre-equilibrated with *n*-heptane. The distributions were carried out in 2-l. separatory funnels (numbered 1–9). At the end of the distribution, funnels 1–4 contained the upper phase and funnels 5–9 the lower phase, with funnel 9 representing the first methanol phase to come in contact with number 1 *n*-heptane. Thirty-eight grams of benzene-extracted wheat flour lipids was dissolved in number 1 heptane and this solution was equilibrated with number 9 methanol. In this first equilibration an emulsion resulted in the lower phase and it was necessary to centrifuge the emulsified phase for 15 minutes at 2000 r.p.m. The clear upper phase was decanted off and added back to number 1 heptane. The lower phase was siphoned off and added to the number 2 heptane funnel. A white interfacial material remained. This was resuspended in 20 ml. of *n*-heptane and filtered by suction on a sintered glass funnel. The residue was washed with another 10-ml. portion of *n*-heptane and the heptane wash was added to number 1 heptane. Number 9 methanol was then equilibrated with number 2 heptane and the process was continued until each of the methanol tubes had been equilibrated in turn with each of the *n*-heptane tubes. In terfacial material resulted only in the first equilibration. On completion of the distribution the solvent was removed from each of the 9 fractions by evaporation at room temperature. As the *n*-heptane was removed from the number 1 tube a steroid crystallized out and was removed by filtration on a sintered glass funnel. These nine fractions were then treated with acetone which resulted in a further fractionation of each into an acetone-soluble and an acetone-insoluble fraction.

An analysis of each of the eighteen fractions as well as of the interfacial material for carbohydrate, phosphorus and nitrogen suggested that at least four distinct fractions were present. All of the acetone-insoluble material as well as the interfacial material contained 2% phosphorus, 6% nitrogen and from 5 to 7% carbohydrate. The acetone-soluble material in the *n*-heptane phase contained less than 1% carbohydrate, and less than 0.2% each of phosphorus and nitrogen while the acetone-soluble material from the methanol phase contained less than 0.5% each of phosphorus and nitrogen but contained approximately 20% carbohydrate.

Since over 80% of the material was present in funnels 1, 8 and 9 in the nine transfer distribution, a second distribution was carried out with only three transfers. One hundred grams of benzene extract was dissolved in 2 l. of *n*-heptane, pre-equilibrated with 95% methanol, in a 4-l. separatory funnel and extracted twice with 1 l. of pre-equilibrated methanol. The first methanol extract was referred to as tube number 3 and the second methanol extract as tube number 2. Tube 1 (heptane phase) contained 62.8 g. of lipid; tube number 2, 10 g.; tube number 3, 21.3 g. In addition 2.9 g. of interfacial material was obtained in the first distribution.

The material from tube number 2 was extracted twice with 150-ml. portions of hot acetone. The residue, consisting of 1.0 g. of lipoprotein, was set aside. The acetone solutions were concentrated to one-half volume and cooled to 0° giving 4.0 g. of white solid which melted at room temperature and resolidified to a white paraffin-like wax (fraction I). The acetone filtrate on removal of the solvent gave 4.0 g. of viscous brown sirup (fraction II). The material from tube number 3 was fractionated in the same way, giving 3.0 g. of lipoprotein, 9.4 g. of fraction I and 7.0 g. of fraction II.

Isolation of Carbohydrate Moieties of Fraction I.—One gram of material in 30 ml. of 0.1 *N* sodium hydroxide was hydrolyzed for 5 hours in a boiling water-bath. Most of the material had dissolved in the first hour. After hydrolysis the reaction mixture was cooled and 0.1 *N* hydrochloric acid was added to a phenolphthalein end-point. These data indicated that 3.42 meq. of alkali was required to hydrolyze 1 g. of the intact lipid.

The alkaline hydrolysate was acidified to *p*H 1.0 with concentrated hydrochloric acid and the fatty acids liberated were extracted with petroleum ether. Removal of the solvent gave 0.578 g. of residue per g. of starting material. If the mean molecular weight of the fatty acids is calculated on the basis of the alkali required for the hydrolysis, a value of 165–170 is obtained. However, when the extracted fatty acids were titrated with alcoholic potassium hydroxide only 1.61 meq. of alkali was required. This discrepancy is undoubtedly due in part to the presence of chlorine in the intact material. The mean molecular weight of the fatty acids based on this latter figure is 359. The nature of these fatty acids is under investigation.

After removal of the fatty acids by extraction with petroleum ether the hydrolysate was deionized by passing the aqueous solution over Amberlite resins IR 120 in the hydrogen phase and IR 400 in the hydroxyl phase. (In subsequent experiments it was necessary occasionally to repeat the treatment with a freshly charged column of IR 400 or

(3) J. K. Dale and C. S. Hudson, THIS JOURNAL, **52**, 2534 (1930).

(4) F. S. Bates, "Polarimetry, Saccharimetry, and the Sugars," United States Government Printing Office, Washington, 1942, p. 718.

(5) E. W. Putman and W. Z. Hassid, THIS JOURNAL, **76**, 2221 (1954).

TABLE III

PAPER CHROMATOGRAPHY OF A AND B[a]

R_f and $R_{Galactose}$ Values

Solvents	A	A hyd.[d]	B	B hyd.[d]	β-Galactosyl-2-glycerol	Galactose	Glycerol
Pyr.:but.:water[b]	0.46	0.46, 0.67	0.23	0.46, 0.67	0.51	0.46	0.67
sym-Collidine[c]	0.95	1.0, 1.58	0.40	1.0, 1.58	0.98	1.0	1.58

[a] Compounds detected with 5% ammoniacal silver nitrate. [b] Pyridine:*n*-butyl alcohol:water (3:2:1.5); R_f values. [c] *sym*-Collidine saturated with water; $R_{Galactose}$ values. [d] Hydrolyzed in 1 *N* hydrochloric acid for 40 min. at 100°.

IR 120 to obtain a neutral solution.) The Molisch and anthrone positive components washed on through the resin columns with little or no holdup. The neutral aqueous solution was lyophilized giving a hygroscopic solid residue.

The procedure of Whistler and Durso[6] was used to isolate the carbohydrate components. This procedure utilizes a carbon–Celite column composed of Darco G-60 and Celite 545 in a 1:1 ratio as the adsorbent. The carbohydrate residue was redissolved in 10 ml. of distilled water. The solution was placed on a 2 × 8 cm. column and elution with water begun. Three hundred ml. of aqueous wash followed by 200 ml. of a 2% ethanol solution yielded no Molisch positive material. Then washing with 5% ethanol was begun, 5-ml. fractions being collected. The carbohydrate content of the fractions was followed by a quantitative anthrone procedure. Two distinct peaks were obtained. The first fraction (A) came off the column in tubes 13 and 20 and the second (B) in tubes 42 to 80. Elution with 95% ethanol did not result in any more Molisch positive material. One gram of fraction I yielded 25–30 mg. of A and 225–270 mg. of B.

Isolation of Carbohydrate Moieties of Fraction II.—This fraction was hydrolyzed with alkali, deionized and the carbohydrate components isolated in exactly the same manner as described above. However, in the elution of the material from the carbon–Celite column, the first fraction was obtained with an aqueous wash while the second fraction required a 2.5% ethanol solution for elution. These two fractions were obtained in about equal quantities. The first fraction to be eluted was shown by the paper chromatographic procedure described below to be identical with the fraction A previously obtained while the second was identical with fraction B.

Crystallization of A and B.—A portion of fraction A was dissolved in a minimum quantity of absolute methanol and stored at 4°. After about two months a significant amount of crystalline material had formed. These crystals were collected and used to seed any subsequent crystallizations. This material could be recrystallized from absolute methanol–ethyl ether mixture although much smaller crystals resulted.

Fraction B was crystallized from an ethanol–water mixture. A portion of this material was dissolved in a minimum quantity of water and absolute ethanol was added until the solution became turbid. The solution was warmed on the steam-cone until it became clear and then allowed to stand at room temperature overnight. Clusters of white needles collected on the walls of the container.

Characterization of A and B.—Paper chromatograms containing up to 150 μg. of fraction A or B gave only one spot when they were sprayed with a 5% ammoniacal silver nitrate spray reagent, dried, and heated in an oven at 110° for 5 minutes. The materials gave reddish-brown spots typical of non-reducing substances. After the materials had been hydrolyzed for 40 minutes at 100° with 1 *N* hydrochloric acid, and subjected to chromatography, two spots appeared with the silver nitrate spray. One corresponded to a glycerol standard and the other to the galactose standard. When the papers were sprayed with aniline hydrogen phthalate only one spot corresponding to the galactose standard appeared. Paper chromatographic data are summarized in Table III.

Glycerol analyses were carried out according to Blix.[7] Both A and B gave mucic acid on treatment with nitric acid. The galactose content was determined quantitatively by the amount of reducing power[7] liberated after hydrolysis in 1 *N* hydrochloric acid at 100° for 1 hour. These data are presented in Table II.

The α-methylphenylhydrazone of galactose[8] was prepared from the acid hydrolysates of A and B. Seventy-three mg. of fraction A was heated in a boiling water-bath for 1 hour with 1 *N* hydrochloric acid. The reaction mixture was then cooled and neutralized with a few drops of concentrated sodium hydroxide. Five ml. of reagent, containing 25 g. of α-methylphenylhydrazine and 3 ml. of glacial acetic acid in 100 ml. of absolute ethanol, was added to the neutralized reaction mixture. The solution was allowed to stand at room temperature for 13 hours and then at 4° for 24 hours. Crystals began to form about 1 hour after the reagent was added. After 24 hours at 4° the crystals were filtered on a sintered glass funnel and washed with 3 ml. of cold absolute ethanol (yield 71.6 mg., m.p. and mixed m.p. 189–191°). *Anal.* Calcd. for $C_{13}H_{20}N_2O_5$: C, 54.90; H, 7.04; N, 9.87. Found: C, 54.04; H, 7.06; N, 9.77. From 100 mg. of crystalline fraction B, 122.6 mg. of the α-methylphenylhydrazone of D-galactose was obtained. When a control containing similar quantities of glycerol and galactose was heated with hydrochloric acid and carried through the same procedure, an 88% yield of α-methylphenylhydrazone of galactose was obtained.

A chromatographic comparison of fraction A with α-D-galactopyranosyl-2-glycerol revealed that the two substances were not identical (see Table III). In addition, fraction A had a specific rotation of +3.77° while α-D-galactopyranosyl-2-glycerol has a specific rotation of +166°.

Periodate Oxidation Studies.—A 0.1-millimole sample of the galactoside was placed in a 50-ml. volumetric flask and water was added to a volume of approximately 25 ml. Five ml. of 0.1 *M* sodium periodate was added and the mixture diluted to the 50-ml. mark. A blank containing the reagents but no galactoside was used. The solutions were allowed to stand at room temperature in the dark for 30 hours. The amount of periodate consumed in the reaction was estimated by removing 5-ml. samples at various time intervals and treating with an excess of 0.05 *M* sodium arsenite in the presence of potassium iodide and sodium bicarbonate and back-titrating with 0.01 *M* iodine. For the determination of formic acid, 5-ml. samples were removed, treated with 0.05 ml. of ethylene glycol and, after 5 minutes, titrated with 0.01 *N* sodium hydroxide using phenol red as an indicator. Formaldehyde was measured by its reaction with chromatropic acid according to Speck and Forist[9] and also by precipitation with dimedon.[10]

The results showed that the monogalactosylglycerol consumed 3.1 moles of periodate, giving rise to 1.0 mole of formic acid and 1.2 moles of formaldehyde. The digalactosylglycerol consumed 5.0 moles of periodate, giving rise to 1.9 moles of formic acid and 1.3 moles of formaldehyde.

Enzymatic Studies.—In these studies a yeast hexokinase preparation[11] contaminated with α-galactosidase activity and a purified β-galactosidase from *E. coli*[12] were used. The α-galactosidase studies were carried out in 0.05 *M* acetate buffer, *p*H 5.0 and the β-galactosidase in 0.1 *M* potassium acid phosphate containing 0.01 *M* methionine. Each enzyme preparation was shown to be specific for its particular type of linkage using melibiose (α-galactoside) and lactose (β-galactoside) as test substrates. The α-galactosidase preparation was also shown to hydrolyze α-D-galactopy-

(6) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

(7) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).

(8) E. L. Hirst, J. K. N. Jones and E. A. Woods, *J. Chem. Soc.*, 1048 (1947).

(9) J. C. Speck, Jr., and A. A. Forist, *Anal. Chem.*, **26**, 1942 (1954).

(10) B. H. Nicolet and L. A. Shinn, *J. Biol. Chem.*, **139**, 687 (1941).

(11) Kindly supplied by Dr. J. Larner, Department of Chemistry, University of Illinois, Urbana, Ill.

(12) Kindly supplied by Dr. S. Spiegelman, Department of Bacteriology, University of Illinois, Urbana, Ill.

ranosyl-2-glycerol to approximately 70% of the theoretical value in 2 hours at 30°.

The enzyme and substrate, in the appropriate buffers, were incubated at 30° and at various time intervals aliquots were withdrawn and deproteinized with barium hydroxide–zinc sulfate. Release of reducing power was determined by the Nelson[8] procedure.

No hydrolysis of the digalactosylglycerol with the β-galactosidase was observed. Incubation with α-galactosidase resulted in the release of only 50% of the potential reducing power. In order to determine the products of this hydrolysis a larger scale experiment was run. Ten mg. of the digalactosylglycerol was incubated with β-galactosidase for 3 hours at 25°. The entire reaction mixture was then deproteinized with barium hydroxide–zinc sulfate. The supernatant solution was put on a 3 × 6 cm. carbon–Celite column (Darco G-60 and Celite 545 in a 1:1 ratio). The column was first washed with 1000 ml. of water and then with approximately 200 ml. of 10% ethanol. These two fractions were concentrated *in vacuo* and finally lyophilized. Paper chromatography of the material from the water wash revealed the presence of a reducing sugar corresponding to galactose. The material from the ethanol wash was non-reducing and was identical with the monogalactosylglycerol of fraction A as judged by paper chromatography. Paper chromatography of an acid hydrolysate of this material revealed the presence of galactose and glycerol.

The monogalactosylglycerol was not hydrolyzed by α-galactosidase during 3 hours incubation at 30°. Incubation with the β-galactosidase resulted in a release of approximately 80% of the potential reducing power in 90 minutes.

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[CONTRIBUTION FROM THE DIVISION OF INDUSTRIAL AND CELLULOSE CHEMISTRY, MCGILL UNIVERSITY, AND THE WOOD CHEMISTRY DIVISION, PULP AND PAPER RESEARCH INSTITUTE OF CANADA]

Attempted Preparation of a Homogeneous Hemicellulose from Aspen Wood[1]

BY J. E. MILKS AND C. B. PURVES

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Chlorite holocellulose from aspen wood (*Populus tremuloides*) was extracted with nearly anhydrous liquid ammonia near 20° and 150 p.s.i. The ammonia removed 10.6% of the wood in the form of acetamide, modified lignin and carbohydrate and rendered a further 9.6% soluble in subsequent extractions with water near 70°. Crude pectins were eliminated from the latter extracts by bleaching with chlorite and by acetylation followed by fractional precipitation. Deacetylation yielded about 1% of the wood as four fractions each containing one hexuronic and two 4-*O*-methylglucuronic acid residues combined with about 22, 16, 13 and 12 anhydro-D-xylose units, respectively. Partial hydrolysis yielded a crystalline aldotriuronic acid containing one 4-*O*-methylglucuronic acid and two xylose residues; also another (uncrystallized) in which one of the xylose residues was thought to be replaced by that of the unknown hexuronic acid. A chemical study by standard methods showed that the hemicellulose was based on anhydro-D-xylose units linked 1–4, probably with many branches in the second and third positions.

Although hemicelluloses extracted from aspen pulps by aqueous alkali have been examined on several occasions,[2-7] the literature records no sustained effort to separate a chemical individual from the original mixture. The present research had the isolation of such a product as one of its objectives, and another was to gain more information about the action of anhydrous, or nearly anhydrous, liquid ammonia on wood pulps. Liquid ammonia at temperatures between 25 and 100° (about 150 to 900 p.s.i.) was known to dissolve only 3.2 and 8.9% of solvent-extracted spruce and maple wood, respectively,[8] but a portion of the maple wood residue was rendered soluble in hot water.[9] The additional solubility was tentatively attributed to the cleavage of ester groups of an unknown type by the liquid ammonia, because the final wood residue contained 0.25% of additional nitrogen as amide and because at least 66% of the acetyl groups in the wood was recovered as acetamide. The acetyl groups in birch and spruce holocelluloses, however, were unaffected when the liquid ammonia was used at atmospheric pressure and −33°,[10a] or under less drastic conditions.

An aspen holocellulose, rather than the wood itself, was used on the present occasion in order to facilitate the isolation of the hemicelluloses. Chlorite holocellulose[11] was preferred to the product obtained by chlorination in water near 0°[12,13] followed by extraction with alcohol–pyridine,[14] because the former method left the holocellulose with a much lower nitrogen content (0.04 %) than did the latter (0.27%).[15] An increase in nitrogen content at this point would complicate the interpretation of any change caused by the subsequent use of liquid ammonia. The chlorite holocellulose (Fig. 1) was then thoroughly extracted with hot water to remove material that might have contaminated the hemicelluloses to be extracted later. This material (extract-1) consisted of polysaccharides and of lignin oxidized in variable degree, but no evidence of a lignin-carbohydrate compound could be found. After being dried, the residual holocellulose was extracted three times under pressure with anhydrous liquid ammonia, which removed 10.2% of the wood

(1) Abstracted from a Ph.D. Thesis submitted by J. E. Milks, October 1953. Presented before the Division of Cellulose Chemistry of the American Chemical Society, Minneapolis, September, 1955.

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(14) G. J. Ritter and E. F. Kurth, *Ind. Eng. Chem.*, **25**, 1250 (1933).

(15) (a) B. B. Thomas, *Paper Ind. and Paper World*, **26**, 1281 (1945); (b) **27**, 374 (1945), noted the increased nitrogen content of chlorine-ethanolamine holocelluloses.