Anal. Calcd. for $C_{12}H_{13}ON_2SCl$: N, 10.43; Cl, 13.20. Found: N, 10.67; Cl, 13.10.

S-(2-Benzoylethyl)-isothiouronium Chloride (IV).—A solution of 7 g. (0.053 mole) of phenyl vinyl ketone in 10 ml. of ethanol was added with shaking to 5 g. (0.066 mole) of thiourea in 30 ml. of 2 *N* hydrochloric acid solution. After ten minutes, 10 ml. of concentrated hydrochloric acid was added, the crystalline precipitate filtered off, dissolved in warm ethanol and reprecipitated by addition of dilute hydrochloric acid. The white crystals were dried at 75°; yield 6 g. (46%), m.p. 143° dec.

Anal. Calcd. for $C_{10}H_{13}ON_2SCl$: S, 13.10; Cl, 14.50. Found: S, 12.91; Cl, 14.30.

4-Phenyl-2-thio-1,2-dihydropyrimidine (IIIa).—When aqueous solutions of Ia or II are allowed to stand for several hours, a yellow solid separates which shows an ultraviolet absorption curve identical with that shown by dilute solutions of Ia or II which have stood for about three hours. The same compound was produced quickly as follows: To a solution of 10 g. of Ia (0.041 mole) in 100 ml. of ethanol and 50 ml. of water was added slowly, with shaking, 3 g. of potassium carbonate. The orange solid which separated was filtered off, washed with water and recrystallized by dissolving in hot ethanol, diluting slowly with water, and cooling. The granular yellow product which separated was filtered off and dried at 75°; yield 5 g. (65%), m.p., dec. > 180°. The compound was insoluble in aqueous acid, but soluble in moderately strong alkali.

Anal. Calcd. for $C_{10}H_8N_2S$: C, 63.80; H, 4.28. Found: C, 64.31; H, 4.07.

1-Benzyl-4-phenyl-2-thio-1,2-dihydropyrimidine (IIIb).—To a solution of 5 g. of Ib in 125 ml. of ethanol was added a solution of 5 g. of potassium carbonate in 15 ml. of water. The two layers were shaken thoroughly, then allowed to stand for one hour at 40 to 50°. The mixture was cooled and the crystalline precipitate was filtered off, recrystallized from hot 95% ethanol and dried at 75°; yield 4 g. (95%), m.p. 160–161°, yellow needles. The compound is insoluble in 1% but soluble in 5% hydrochloric acid solution.

Anal. Calcd. for $C_{17}H_{14}N_2S$: C, 73.35; H, 5.07; N, 10.08. Found: C, 72.91; H, 5.02; N, 10.50.

Ultraviolet Absorption Spectra.—The spectrophotometric studies were carried out with a Cary recording quartz spectrophotometer, model 11, serial no. 37, with a voltage setting of 4 and a slit control setting of 20. Quartz cells of 10 mm. thickness were used and concentrations were adjusted (10 to 30 mg. per liter) so as to give optical density readings in the range of 0.5 to 2.0.

Acknowledgments.—We are indebted to M. E. Auerbach, Kenneth Fleischer and staff for the microanalytical data, and to Drs. C. F. Koelsch and E. J. Lawson for comments on the reaction mechanisms.

RENSSELAER, N. Y. RECEIVED OCTOBER 28, 1950

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, JOSEPH E. SEAGRAM & SONS, INC.]

Isolation of a Crystalline Trisaccharide from the Unfermentable Carbohydrate Produced Enzymically from Maltose[1]

BY S. C. PAN, L. W. NICHOLSON AND PAUL KOLACHOV

Submerged cultures of *Aspergillus niger* NNRL 337 contain an enzyme(s) which converts maltose into an unfermentable carbohydrate(s) in addition to glucose. The fermentable sugars (glucose and maltose) are removed from the unfermentable carbohydrate by yeast fermentation; other impurities are removed by basic lead acetate precipitation and by passing the filtrate through ion-exchange resins. A crystalline product is readily obtained from an aqueous methanol solution containing the purified unfermentable carbohydrate. The twice recrystallized product was shown to be a trisaccharide on the basis of its reducing power toward NaIO, its molecular weight, and its paper chromatography. The following evidence shows that the trisaccharide consists of only glucose residues: reducing power of the acid hydrolysate of the trisaccharide toward NaIO; complete fermentability of the hydrolysate; and failure of the hydrolysate to give insoluble phenylhydrazone.

The rate at which the trisaccharide crystallizes from the aqueous methanol solution increases as the temperature is increased (up to the boiling point of the solution). When the fungal culture used in the synthesis has been stored in the refrigerator the rate of synthesis increases but the yield of the crystalline product decreases as the storage time increases.

Introduction

The discovery that submerged cultures of *Aspergillus niger* (NRRL[2] 337) contain an enzyme(s) which can synthesize an unfermentable carbohydrate(s) from maltose has been reported previously.[3] The unfermentable carbohydrate was first obtained as a precipitate; its reducing power showed that it was an oligosaccharide containing not more than three glucose residues per molecule. Further investigation has led to the isolation of a crystalline material from the unfermentable carbohydrate. The purified crystalline product has been shown to be a trisaccharide, containing three glucose residues per molecule.

Norberg and French,[4] using electrophoretic technique, have recently shown that *Bacillus macerans* amylase is capable of converting maltose into a trisaccharide and higher oligosaccharides. The enzyme reaction of *A. niger*, discussed in this paper, will certainly be very similar to that of *B. macerans*. None of the oligosaccharides, however, reported by Norberg and French have been crystallized. It is not known, as yet, whether the trisaccharide described in this paper is one of the products obtained by the action of *B. macerans* amylase acting upon maltose.

The occurrence of trisaccharides composed only of glucose residues as the end products of the enzymic hydrolysis of starch, has been reported by Blom and Rosted[5] and by Myrbäck and associates.[6] The products they describe are not crystalline and, therefore, the exact chemical nature of these sugars has not been fully proved. Recently, Wolfrom and associates[7] have isolated maltotriose as its hendecaacetate in crystalline form from an enzymic

(1) Presented at the 118th Annual Meeting of the American Chemical Society at Chicago, Ill., on September 6, 1950.

(2) Northern Regional Research Laboratory.

(3) S. C. Pan, A. A. Andreasen and Paul Kolachov, *Science*, **112**, 115 (1950).

(4) E. Norberg and D. French, THIS JOURNAL, **72**, 1202 (1950).

(5) J. Blom and C. O. Rosted, *Acta Chem. Scand.*, **1**, 233 (1947).

(6) K. Myrbäck, *Biochem. Z.*, **297**, 179 (1938); K. Myrbäck, *Advances in Carbohydrate Chem.*, **3**, 251 (1948); B. Ortenblad and K. Myrbäck, *Biochem. Z.*, **303**, 335 (1940).

(7) J. M. Sugihara and M. L. Wolfrom, THIS JOURNAL, **71**, 3357 (1949); M. L. Wolfrom, L. W. Georges, Alva Thompson and I. L. Miller, *ibid.*, **71**, 2873 (1949).

hydrolysate of starch; the free sugar has not been crystallized. The trisaccharide reported here, therefore, is the first one of this group of glucose polymers to be isolated in crystalline form.

The procedures employed for the synthesis and purification of the unfermentable carbohydrate were essentially the same as those previously reported.[1] The few modifications that were made will be discussed later.

The trisaccharide was readily crystallized from an aqueous methanol solution (I) containing the purified unfermentable carbohydrate in much the same manner as glucose or maltose is crystallized. A yield of crystalline product equal to 70% of the unfermentable carbohydrate in (I) was obtained.

Use of freshly prepared fungal cultures for the synthesis, and maintaining the proper temperature during crystallization were found to be requisites for obtaining the crystalline product rapidly and efficiently. The rate of crystallization increased markedly as the temperature was increased. This may appear to be inconsistent with the general concepts of crystallization; actually, it is in agreement with known theories dealing with kinetics of crystallization[8] provided the solubility of the solute or the degree of supersaturation is not appreciably affected by temperature.

When fungal cultures used in the synthesis were stored in the refrigerator the rate of synthesis increased, but the yield of crystalline product that could be obtained decreased markedly as the storage time increased. This suggests that during storage some other enzyme(s) may be activated which can synthesize still another unfermentable carbohydrate. Further investigation is required before this explanation can be substantiated.

The trisaccharide crystallizes from a 15% aqueous methanol solution (water to methanol = 1:2) in thin prisms (Fig. 1). Other fundamental properties of the twice recrystallized product (III) are summarized in Table I.

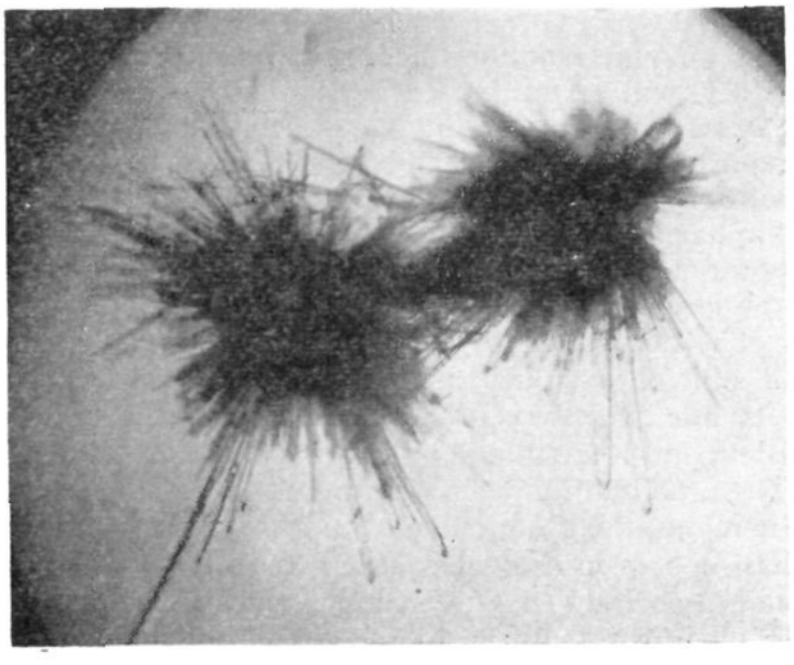

Fig. 1.—Photomicrograph of the crystalline trisaccharide.

(8) J. H. Perry, "Chemical Engineering Handbook," 3rd Ed., McGraw-Hill Book Co., Inc., New York, N. Y., pp. 1054, 1059; A. Van Hook, *Ind. Eng. Chem.*, **36**, 1042 (1944); A. Van Hook, "The rate of crystallization of sucrose," S. R. F. Project No. 7, Sugar Research Foundation, Inc., 1949.

TABLE I

PROPERTIES OF THE CRYSTALLINE PRODUCT

Properties		Theoretical values	Remarks
Tests on air-dried sample			
Crystalline form	Thin prisms		Fig. 1
Loss at 55° under 28 to 29 in. vacuum, %	0.51		
Loss at 105 to 110°, %	0.51		Anhydrous
Exposure to air	Non-hygroscopic		
Ash, %	0.09		
Molisch test	Positive		
Phenylosazone test	Negative		
Fermentability	Not by bakers' or distillers yeast but fermented by *Schizosaccharomyces pombe*		
Tests on oven-dried sample			
M.p., °C.	213 (dec.)		
$[\alpha]^{25}D$ (c, 2.2; l, 4; water)	+154° (mutarotation downward)		
Reducing power toward copper–tartrate–phosphate reagent, calculated as glucose, %	40		
Reducing power toward NaIO, calculated as glucose, %	35.4	35.7	
Carbon, %	43.29	42.86	
Hydrogen, %	6.49	6.40	
Molecular weight (by f.p. depression)	475	504	
Paper chromatography			See text
Tests on acid hydrolysate			
Total reducing power toward copper–tartrate–phosphate reagent, calculated as glucose, %	103.5	107.1	
Total reducing power toward NaIO, as glucose, %	103.0	107.1	
Fermentability	Completely fermented by bakers' yeast at *p*H 8.4 in 20 minutes		
Phenylhydrazine test	No insoluble phenylhydrazone but formation of glucosazone		

The vacuum oven-dried sample lost no additional weight when heated at 105 to 110°, showing that the crystals were anhydrous; the high melting point supported this conclusion. The positive Molisch test and the fermentability by *Schizosaccharomyces pombe* show that the crystalline product is a carbohydrate while the negative phenylosazone test confirms the conclusion that it is an oligosaccharide higher than maltose.

The results of the following tests indicate that the crystalline product is a trisaccharide: (1) reducing power toward sodium hypoiodite; (2) molecular weight, determined by freezing point depression of water; and (3) comparison with glucose and maltose by paper chromatography. The relative distances through which glucose, maltose, and the crystalline trisaccharide migrated are in the ratio of 14:10.5:5.8, respectively.

That the trisaccharide consists only of glucose residues is based on the following evidence: (1) the close agreement between the reducing power

of the acid hydrolysate[9] toward copper–tartrate–phosphate reagent and that toward sodium hypoiodite, indicating the absence of ketoses; (2) the complete disappearance of reducing power after hydrolysis and treatment with bakers' yeast for twenty minutes at *p*H 8.4—indicating the absence of galactose and probably mannose; (3) failure to obtain an insoluble phenylhydrazone from the hydrolysate (when cold) while a glucosazone was formed when heated—a further indication that no mannose is present.

Experimental

Submerged Fungal Culture.—A submerged culture of *Aspergillus niger* NRRL 337[2] was prepared in a medium containing the following ingredients per liter: corn 20 g., ammonium sulfate 2 g., potassium dihydrogen phosphate 1 g., magnesium sulfate 0.5 g., ferrous sulfate 0.01 g. and calcium carbonate 5 g. This semi-synthetic medium was used instead of the corn and distillers dried solubles medium used in previous studies,[3] because it introduced less impurities, making the subsequent purification simpler and more efficient. The medium was divided into 120-ml. portions, placed in 750-ml. erlenmeyer flasks, sterilized, and inoculated with spores of the mold which had been grown on agar slants of Czapek medium for one week. The inoculated medium was incubated for 48 to 66 hours at 30° on a shaker operating at 85 cycles per minute with a stroke length of two inches.

Analyses for Carbohydrate.—Mixtures of glucose, maltose and unfermentable carbohydrate were analyzed using the method of Stark and Somogyi.[10] All sugar values were expressed as glucose equivalents and were determined with Somogyi's copper–tartrate–phosphate reagent[11] after hydrolyzing the samples in 0.69 *N* hydrochloric acid in a boiling water-bath for 2.5 hours.

The reducing power of the sugars toward sodium hypoiodite was determined by the method of Caldwell, Doebbeling and Manian.[12]

Enzymic Synthesis.—One volume of filtrate from the fungal culture was added to two volumes of a buffered maltose solution. The resulting mixture contained approximately 100 mg. of maltose per ml. and 10 ml. of McIlvaine's standard buffer (*p*H 4.5) per 100 ml. A small amount of toluene was added and the reaction mixture was incubated at 50°; this relatively high incubation temperature was used in an effort to check contamination. The progress of synthesis is shown in Fig. 2. Over 50 mg. of unfermentable carbohydrate and 26 mg. of glucose per ml. were produced in 72 hours; at the same time the maltose concentration was reduced from 105 mg. to 28 mg. per ml.

When the fungal culture is propagated in the semi-synthetic medium the enzyme systems are apparently different from the ones which are produced when the fungal culture is propagated on a corn and distillers dried solubles medium. The amount of glucose formed during synthesis is considerably less when a fungal culture propagated on a semi-synthetic medium is used (*cf.* previous report[3]). The reaction was stopped at the end of 72 hours by autoclaving.

Removal of Fermentable Sugars by Yeast Fermentation.—A sterilized yeast nutrient solution,[13] equal to one-tenth the total volume, was added to the sterilized reaction mixture. The resulting medium was inoculated with an actively growing culture of distillers yeast (Seagram strain 1). The addition of the yeast nutrients assured the complete removal of glucose and maltose after four days of incubation at 30°.

Purification of the Unfermentable Residue.—The fermented medium contained approximately 40 mg. of the unfermentable carbohydrate per ml.; yeast cells were removed by decantation. The supernatant solution was treated with basic lead acetate and filtered. The filtrate

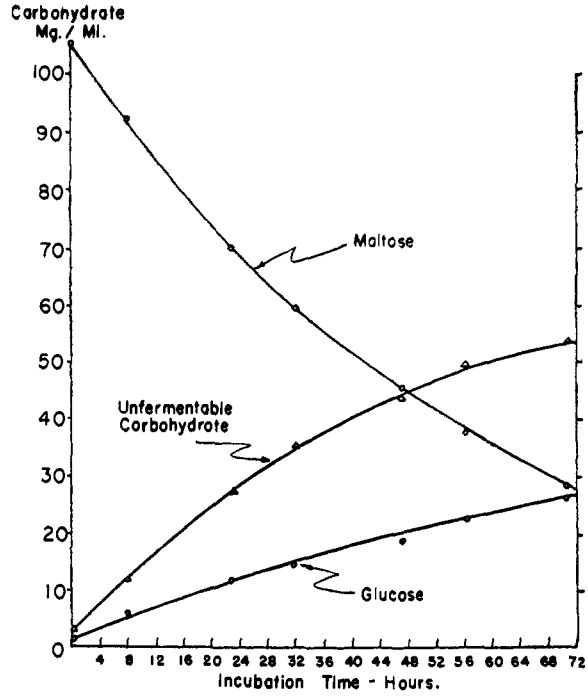

Fig. 2.—Enzymic synthesis of unfermentable carbohydrate from maltose.

was passed twice alternately through beds of ion-exchange resins, Amberlite IR-100 and IR-4B. The clear, colorless effluent was concentrated under reduced pressure to a thick sirup containing 0.8 to 0.9 g. of the unfermentable carbohydrate per ml. Methanol, equal to four times the volume of the sirup, was added slowly with constant stirring. Any precipitate that formed during the addition of the methanol could be dissolved by adding small amounts of water; traces of turbidity were completely removed by centrifugation. Storing the methanol solution in the refrigerator overnight facilitated the removal of turbidity.

Crystallization.—Crystals of the trisaccharide (II) quickly formed when the clarified aqueous methanol solution (I) was kept in a tightly closed bottle at, or above, room temperature (25–30°). Crystallization can be hastened by seeding the methanol solution. The crystals were collected by filtration, washed with methanol and dried. Seventy per cent. of the unfermentable carbohydrate present in the methanol solution (I) could be recovered as crude crystals (II) (90 g. from 400 g. of maltose used for synthesis). The crystals (II) were purified by recrystallization from aqueous methanol; the recovery amounted to 85 to 90%. Twice recrystallized product (III) was used for characterization studies.

Effect of Temperature upon the Rate of Crystallization.—The clarified aqueous methanol solution (I) was maintained at three different temperatures, 25, 30 and 70°. The 70° temperature was obtained by boiling the methanol solution (I) under reflux. During crystallization weighed samples were taken of the supernatant mother liquor (centrifuged, if necessary), and analyzed for carbohydrate (as glucose after acid hydrolysis). The percentage of crystallization was calculated from the change in weight of carbohydrate per unit weight of solvent. The results, summarized in Table II, show that the rate of crystallization increases markedly as the temperature at which crystallization takes place is increased.

TABLE II

RATE OF CRYSTALLIZATION AT DIFFERENT TEMPERATURES

Temp. of cryst., °C.	Per cent. crystallized in 8 hr.	24 hr.	48 hr.	7 days	45 days
2–5	..	..	..	..	32.0
30	..	21.3	50.0	68.8	69.5
70 ± [a]	70.1	70.2	..	..	..

[a] Boiling temperature of the aqueous methanol solution.

(9) A 1% solution of the trisaccharide was hydrolyzed in 0.69 *N* hydrochloric acid in a boiling water-bath. Maximum reducing power was reached after 2.5 hours of hydrolysis.

(10) I. E. Stark and M. Somogyi, *J. Biol. Chem.*, **142**, 579 (1942).

(11) M. Somogyi, *ibid.*, **160**, 61 (1945).

(12) M. L. Caldwell, S. E. Doebbeling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

(13) B. H. Olson and M. J. Johnson, *J. Bact.*, **57**, 235 (1949).

Effect of Storing the Fungal Culture in the Refrigerator upon Yield of Crystalline Product and Rate of Synthesis.—The fungal culture was stored in the refrigerator and samples were withdrawn at weekly intervals and used for synthesis experiments. The yield of crystalline product obtained by boiling the aqueous methanol solution (I) for eight hours was determined. Figure 3 shows that the yield of crystalline product decreased markedly as the length of storage time increased. Practically no crystals were obtained when fungal cultures which had been stored for five weeks were used for the synthesis.

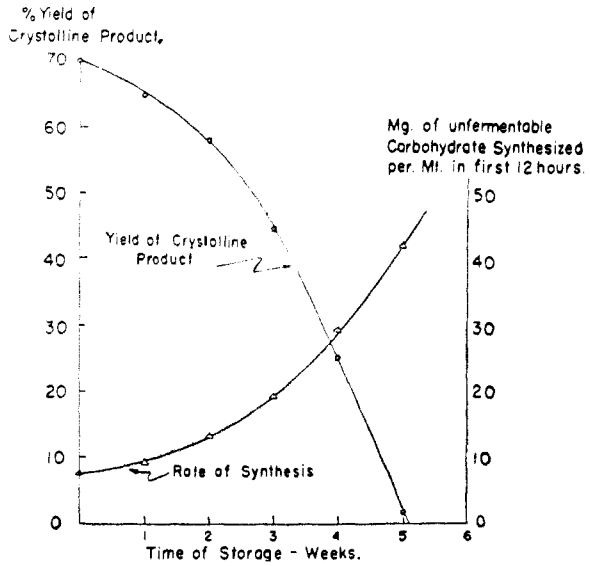

Fig. 3.—Effect of storing the fungal culture upon rate of synthesis and yield of crystalline product.

On the other hand, the rate of synthesis, as determined by the mg. of unfermentable carbohydrate synthesized in the first 12 hours of incubation (*cf.* Fig. 2), increased markedly as the length of storage time increased (Fig. 3).

Because the unfermentable carbohydrate synthesized tended to disappear after all the maltose had been consumed,[3] the maximum amount that could be synthesized could hardly exceed 60 mg. per ml. When the fungal culture which had been stored in the refrigerator for three weeks or more was used for the synthesis, this limit (60 mg. per ml.) was reached in less than 72 hours and the reaction had to be stopped correspondingly earlier. The possibility that the synthesis had already proceeded beyond the limit before it was stopped, however, could not be excluded. Whether the yield of crystalline product was affected by over incubation was not determined.

Paper Chromatography.—A modification of the apparatus described by Ma and Fontaine[14] was used for preparing ascending-type paper chromatograms. The elution solvent, a mixture of pyridine, butanol and water (2:3:1.5, respectively) and the developing agent, a 0.5% solution of 3,5-dinitrosalicylate in 4% sodium hydroxide, were developed by Dr. R. J. Dimler and associates of the Northern Regional Research Laboratory.

A 10% solution of the sugar was spotted five times on a strip of Whatman No. 1 filter paper and eluted for sixteen hours at room temperature (25 to 30°). The strip was air-dried, sprayed with the developing agent, air-dried again, and heated at 105° for 10 minutes.

Acknowledgments.—The authors wish to thank Dr. M. L. Wolfrom of Ohio State University for his helpful advice and Dr. R. J. Dimler of Northern Regional Research Laboratory for the paper chromatographic method.

(14) R. M. Ma and T. D. Fontaine, *Science*, **110**, 232 (1949).

LOUISVILLE, KY. RECEIVED SEPTEMBER 18, 1950

[CONTRIBUTION FROM THE PULP MILLS RESEARCH PROJECT AND THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING OF THE UNIVERSITY OF WASHINGTON]

Non-degradative Oxidation of Lignin Sulfonates

BY D. M. RITTER

In order to obtain information concerning the non-benzenoid portion of lignin derivatives, sodium peracetoxy lignin sulfonate was oxidized with mercuric acetate, quantitatively and without molecular degradation. The product was a carboxy dihydroxylated lignin sulfonate from which methylated and acetylated derivatives and a potassium salt were prepared. The product and its methylated derivatives underwent spontaneous γ-lactone formation. It was concluded that each C_{18} unit of the lignin sulfonate ion contains at least four contiguous aliphatic carbon atoms, the terminal one of which bears the hydroxyl group of a primary alcohol. The fourth carbon in the group may bear one of the aromatic rings.

Oxidation of basic lignin sulfonate solutions with metallic oxides has contributed substantially to an understanding of the aromatic character of lignin derivatives and of the substituents in the ring.[1] However, those obviously complex and multiple reactions give products which until now have offered little information concerning the aliphatic portions of lignin. The present study of the action of mercuric acetate on lignin sulfonate derivatives furnishes more experimental information concerning the non-benzenoid parts of the lignin structure.

When a lignin sulfonate salt in 1 *N* acetic acid solution was treated with mercuric acetate at room temperature, two reactions were observed. One of these was a mercuration,[2] and the other was an oxidation characterized by the slow separation of mercurous acetate. The rate of oxidation was increased to the useful point and the mercuration was suppressed by performing the reaction with sodium peracetoxy lignin sulfonate dissolved in a boiling solution of sodium acetate and mercuric acetate in glacial acetic acid. In the 24 hours required for complete reaction no methoxyl loss occurred[3] nor was degradation in molecular size observed as shown by the diffusion constants, D = 7.1 mm.²/day, before the reaction, and D = 7.2 mm.²/day, after the oxidation.

The oxidation product (I) and its derivatives had compositions given in Table I. The potassium sulfonate-carboxylate salt (II) was water soluble, but the barium salt was practically insoluble. After repeated treatment of (I) with diazomethane[4,5] the

(1) W. Lautsch, E. Plankenhorn and F. Klink, *Angew. Chem.*, **53**, 450 (1940); I. A. Pearl, THIS JOURNAL, **64**, 1429 (1942); **71**, 2196 (1949); **72**, 1427, 2309 (1950).

(2) The mercuration reaction, which has been studied by Dr. K. A. Wright, will be the subject of a separate communication.

(3) *Cf.* Table III.

(4) E. G. King, F. Brauns and H. Hibbert, *Can. J. Research*, **13B**, 88 (1935).

(5) D. M. Ritter, E. D. Olleman, D. E. Pennington and K. A. Wright, THIS JOURNAL, **72**, 1347 (1950).