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The Oxidation of a Spruce Glucomannan With Lead Tetraacetate

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THE OXIDATION OF A SPRUCE GLUCOMANNAN WITH LEAD TETRAACETATE

A thesis submitted by

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SUMMARY

The purpose of this thesis was to oxidize a well-characterized glucomannan with lead tetraacetate, and to study the distribution of glucose units along the polymer chain through the isolation of residual oligosaccharide units rich in glucose.

The source of the glucomannan was a white spruce sulfite pulp, containing 12.7% mannose units, which was prepared by a two-stage pulping process (31-33). The polymer was isolated by a novel extraction and precipitation procedure which involved selective precipitation of the glucomannan from alkali-borate solution by the addition of barium acetate. The purified glucomannan contained only mannose, glucose, and galactose units in the ratios 3.25:1:0.05. The polysaccharide was homogeneous by electrophoresis, had a number average degree of polymerization of 70, an intrinsic viscosity of 0.34 in molar cupriethylenediamine, and a specific rotation of -34° in aqueous alkali.

Studies of the oxidation of the glucomannan with lead tetraacetate were conducted in aqueous acetic acid. The reaction variables studied were (a) water concentration (b) polymer concentration and (c) initial mole ratio of oxidant to sugar unit. An increase in any of these variables increased the rate of oxidant consumption, but they had little effect on the degree of selectivity of the oxidation for the mannose units. Under all conditions, the mannose-to-glucose ratio was reduced from 3.2 to 1.5 and then was stable. The only way that this stable ratio could be lowered was by reducing the oxidized polymer with sodium borohydride and subjecting this material to a second oxidation.

It was also found that, unlike periodate, measurement of the consumption of lead tetraacetate by a glucomannan is not a useful tool for predicting the structure of the polymer.

The oxidized glucomannan was hydrolyzed selectively with sulfurous acid. In addition to glucose, mannose, and the oxidation fragments, two disaccharides, cellobiose and 4-O-β-D-glucopyranosylmannose, were found in identifiable quantities. Paper chromatography indicated the presence of mannopyranosylglucose in trace amounts, but the presence of mannobiose was questionable. No evidence of oligosaccharides of higher degrees of polymerization was found.

The evidence obtained was accepted as proof that contiguous glucose units existed in the glucomannan as cellobiose units, and that this disaccharide was not the degradation product of a polyglucan contaminant. However, the amount of cellobiose was much smaller than should have been recovered for a purely random distribution of glucose units.

Finally, a hypothesis was formulated to explain the resistance of some of the mannose units to oxidation with lead tetraacetate. The essence of this hypothesis is that the resistant mannose units were protected from selective oxidation by an adjacent oxidized unit. To account for this stabilization, a possible mechanism, based on the formation of a hemiacetal linkage between an aldehyde group of an oxidized unit and the nearest hydroxyl group of the unoxidized neighbor, was proposed.

INTRODUCTION AND ANALYSIS OF PROBLEM

The glucomannans are the most important hemicellulose components in softwoods where they account for 15-20% of the dry weight of the wood (1). The glucomannans isolated from a large number of softwood species all contained similar mannose-to-glucose ratios of about 3 to 1 (2-14). Smaller amounts of galactose units are usually found in these polymers also.

One of the most intriguing problems in the structural study of the glucomannans is the order of linkage between the mannose and glucose units in the polymer. Previous studies have shown that both sugar units are bound together in the polymer backbone by β-1-4 linkages, but whether they are linked in some regular repeating unit or purely at random has not been determined.

Graded acid hydrolysis has been used to provide much information on this linkage order. Several investigators have succeeded in isolating the homologous β-1-4 mannose oligosaccharide series through mannotetraose (12, 13, 15, 16). Conclusive evidence has also been obtained for the chemical linkage between mannose and glucose units in the polymer. This was done through the isolation of 4-O-β-D-glucopyranosyl-D-mannose and 4-O-β-D-mannopyranosyl-D-glucose. Trisaccharides containing both mannose and glucose have also been isolated (15, 17).

Much less is known about the possible contiguous nature of the glucose linkages. Although cellobiose has been found in the hydrolyzates of several glucomannans (6, 13, 15), the amounts isolated are too small to definitely establish this linkage in the glucomannans. Small amounts of a glucan contaminant could easily account for all of the cellobiose found thus far.

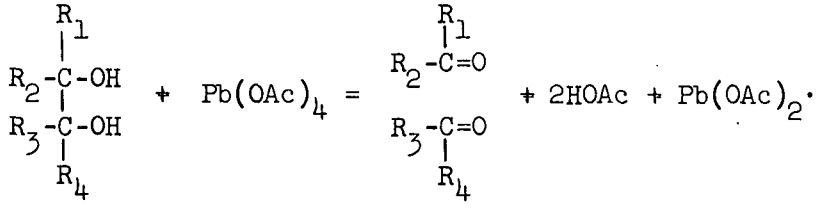
The best evidence for the existence of contiguous glucose units in the glucomannans has been the isolation of the glucopyranosyl-glucopyranosyl-mannose and mannopyranosyl-glucopyranosyl-glucose trisaccharides (15, 17). However, these trisaccharides were isolated from highly soluble hemicellulose fractions with anomalous sugar unit ratios not representative of the main body of the glucomannans.

The study of the possible linkages between glucose units by graded acid hydrolysis is complicated by the fact that mannose units constitute 75-80% of the glucomannan. The preponderance of mannose-containing oligosaccharides in the hydrolyzate tends to mask the glucose-containing oligosaccharides and make their separation and identification difficult. For this reason, any reaction which would destroy the mannose units selectively prior to hydrolysis and separation of the oligosaccharides should be a valuable tool for the study of these glucose linkages.

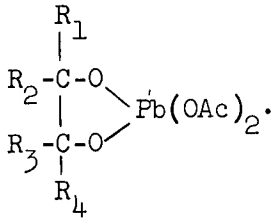
In mannose, the 2-epimer of glucose, the vicinal (vic) hydroxyls on the 2,3-carbon atoms are in the cis glycol configuration. Glucose has a 2,3-trans-glycol configuration. This difference in structure causes differences between the two sugars in response to reactions involving the glycol group.

Lead tetraacetate, like periodate, cleaves oxidatively vic glycol groups. In addition, lead tetraacetate in acetic acid solution is especially suitable for differentiating between various kinds of glycol groups.

Criegee (18) illustrated the oxidation reaction as follows:



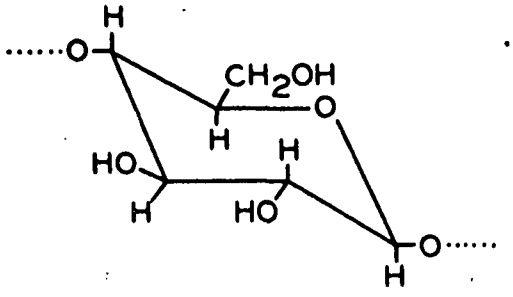
It was postulated that the reaction proceeded through the following cyclic intermediate:



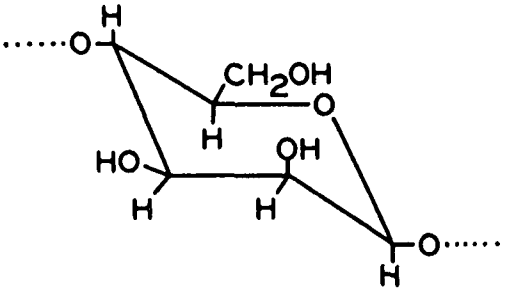
This original proposal has been modified recently (19) to avoid the necessity of the five-membered transition ring form in order to explain anomalous reaction rates of certain materials. However, the cyclic intermediate appears to be the major reaction pathway with the sugars.

Dimler (20) has stated that the necessity for both hydroxyls to be engaged in a co-ordination complex before oxidative cleavage can occur would account for the difference in rate of oxidation of cis- and trans-glycols. However, the actual distance between the hydroxyl groups appears to have a relatively minor effect on the rate of oxidation by lead tetraacetate (21). Reeves (22) has shown that coplanarity of the hydroxyls is of much greater significance for glycol-complexing. That is, the closer the two hydroxyl groups approach each other in a plane with the adjacent carbon atoms, the more favorable the arrangement for complexing. True cis-glycol groups have carbon-oxygen bond pairs which subtend an angle of 0°. Such glycols complex readily with lead tetraacetate and, consequently, are oxidized very rapidly.

In β-1,4 polymers, mannose and glucose units exist in the pyranose ring form. The preferred conformation of pyranose rings is the stable chair form shown in the following structures (22, 23).



β-D-Glucose Unit



β-D-Mannose Unit

True cis or trans orientation does not exist in this structure. The 2,3 hydroxyls of the glucose unit have an axial-axial "trans" orientation in which the bonds subtend a 60° angle. The 2,3 hydroxyls of the mannose unit have an equatorial-axial "cis" orientation in which the bonds also subtend a 60° angle. Although these angles are equal, deformation about the carbon-carbon bond to provide the coplanarity required for lead tetraacetate complexing causes decreased ring puckering in the cis form, but increased ring puckering in the trans form. Greater energy must be expended to deform the trans isomer; therefore, its oxidation rate should be noticeably lower than that of the cis isomer.

Perlin has reviewed the literature on the action of lead tetraacetate on carbohydrates (24). He reports that in all oxidations of monosaccharides, glycosides, and oligosaccharides studied, the rates of oxidation are greater for the cis than for the trans isomers. In addition, he found that oligosaccharides of the xylobiose, cellobiose, and maltose homologous series contained glycol groups which are resistant to oxidation (25). These homologues all contain 2,3-trans glycols and are linked through positions 1,4. In these investigations the oligosaccharides of a particular series consumed equal amounts of oxidant regardless of the degree of polymerization. The amount consumed corresponded to that required for oxidation of the end groups alone. Perlin suggested that the

end groups are sensitive to oxidation regardless of configuration, but that the 2,3-trans glycols of central residues in 1,4-linked polymers are resistant to oxidation by lead tetraacetate.

Apparently, the glycol group is held in too rigid a trans orientation for an adequate approach to the coplanarity required for oxidation. Perlin also suggested that central mannose residues probably would not resist oxidation because their 2,3-cis glycols could be forced into coplanarity more easily.

Steinmann and White (26) and Roudier and Nick (27) have investigated the lead tetraacetate oxidation of wood pulps in acetic acid systems. Both investigations indicated that mannose units were oxidized to a greater degree than are other sugar units present in the pulp. However, Roudier and Nick concluded that the oxidation was not specific enough for the determination of mannose units in pulps.

A subsequent investigation of the oxidation of wood pulps by Matsuzaki and Ward (28) indicated that although a correlation between content of mannose units and consumption of lead tetraacetate existed for various pulps, the mannose itself was not lost to the extent which one would expect if the process were selective for mannose units. These investigators stated that variations in the initial consumption of oxidant between various pulps might be better correlated with the accessibilities of the pulps or with the number of end groups in the pulps than with mannose content.

More recently, Detrick (29) conducted a statistical study of the oxidation on well-characterized pulps. The study was planned to include information on the rate of oxidation with respect to:

1. The mannose unit content of the pulp.
2. The xylose unit content of the pulp.
3. The accessibility of the pulp.
4. The number of end groups in the pulp.

He concluded that the mannan content of the pulp is the primary factor influencing the oxidation.

In all of these studies, the quantities of lead tetraacetate consumed were very low. The largest amount of oxidant consumed in any of these investigations was only 0.05 mole of lead tetraacetate per mole of anhydrosugar unit. In addition, only about 60% of the mannose units in the pulps could be oxidized. Apparently, factors other than resistant trans-glycol groups and accessibility, as measured by Detrick, played an important role in the oxidation reaction.

From the above discussion, it is logical to assume that in the extensive oxidation of an accessible glucomannan with lead tetraacetate the mannose units would be attacked preferentially. Selective degradation of such a material would yield oligosaccharides enriched in glucose. Through the isolation and identification of such oligosaccharides, information about the contiguous nature of the linkages between glucose units might be obtained.

The experimental program of this thesis was designed to test this hypothesis. The investigation included the isolation and characterization of a softwood glucomannan, the comprehensive study of the oxidation of this glucomannan with lead tetraacetate, selective hydrolysis of the oxidized polymer, and the identification of certain of the hydrolysis products. In addition, the study was enlarged in an attempt to explain the unexpected resistance of some of the mannose units toward oxidation.

EXPERIMENTAL

PAPER CHROMATOGRAPHY

Paper chromatography of sugars was conducted on Schleicher and Schuell no. 598 paper unless noted otherwise. All papers used for quantitative estimations were dipped in distilled water and dried without tension prior to use. The papers were irrigated with one of three solvents: A, ethyl acetate–pyridine–water (8:2:1); B, butyl acetate–pyridine–95% ethanol–water (8:2:2:1); and C, ethyl acetate–acetic acid–water (9:2:2).

Spots were developed by dipping the papers in a solution of 0.5% aniline and 1.5% monochloroacetic acid in anhydrous ether and heating for 5-8 minutes at 100°C. (30). This reagent was sensitive to 1 μg. of monosaccharide and 2 μg. of disaccharide.

GLUCOMANNAN ISOLATION

SOURCE MATERIAL

Six freshly cut white spruce (Picea glauca) logs were secured from the Kimberly-Clark Corporation. These logs were cut from different spruce stands in order to provide maximum variation between individual trees. The logs were chipped and blended to obtain a representative sample. Uniformity of sample was also enhanced by pulping only those chips which passed a 3/4-inch mesh screen and were retained on 1/4-inch mesh.

PULPING PROCEDURE

The pulping procedure was based on the glucomannan stabilization work of Annergren and Rydholm (31-33). It was designed to prepare a pulp in high yield

which contained large amounts of glucomannan with a low percentage of galactose units. The procedure involved a unique two-stage neutral sulfite–acid sulfite cook.

The following cooking schedule was used to treat 7285 g. of sprucewood chips in two equal batches.

Stage one:	
Maximum temperature, °C.	130
Time to maximum temperature, hr.	3/4
Time at maximum temperature, hr.	1-1/2
Liquor concentration, SO_2 by wt., %	1
Initial pH of liquor	8.0
Stage two:	
Maximum temperature, °C.	140
Time to maximum temperature, hr.	1/4
Time at maximum temperature, hr.	1
Liquor concentration, SO_2 by wt., %	4
pH at beginning of stage two	1.8-2.0

The liquor for the first stage was prepared by dissolving sufficient sodium sulfite in tap water to give a 1% solution calculated as sulfur dioxide. The pH of this solution was adjusted to 8.0 by bubbling a small amount of gaseous sulfur dioxide into the freshly prepared liquor.

At the beginning of the second stage, the liquor concentration was increased to 4% total sulfur dioxide by injecting a weighed amount of liquid sulfur dioxide directly into the digester. This injection was accomplished without cooling between stages through the use of nitrogen at 120 p.s.i.

At the end of the second stage, the digester was blown, and the chips were defibered on a Sprout-Waldron disk refiner. The pulp was washed, screened on a vibrating flat bed screen, air dried, and Wiley milled to pass 10 mesh. After milling, the two batches of pulp were combined and stored in polyethylene bags at 4°C. until used.

A total of 5405 g. of ground pulp was recovered. This represents a yield of 74.3% of the wood.

The pulp was characterized by several standard methods. Alcohol-benzene solubility was determined by Institute Method no. 11. Glucose, mannose, and xylose contents were determined by the method of Saeman and co-workers (34) modified by the use of barium hydroxide in lieu of ion-exchange resin to neutralize the acid. Galactose and arabinose were also present in trace amounts but were not determined quantitatively. Lignin was determined as the insoluble residue remaining after the Saeman hydrolysis of the pulp. The analytical results are shown in Table I.

TABLE I

SPRUCE PULP ANALYSIS

	Per Cent of Pulp
Alcohol-benzene solubles	1.2
Lignin	20.6
Glucan	55.0
Mannan	12.7
Xylan	5.6
Total	95.1

The mannan yield of this pulp was much higher than from a normal acid sulfite cook (31).

GLUCOMANNAN EXTRACTION

The procedure described in this section was designed specifically to isolate large quantities of pure glucomannan with minimum volumes of solvent.

Quantitative removal of the glucomannan from the pulp was not sought. Purity of the fraction was the important criterion. This procedure represents a radical departure from the methods of glucomannan isolations reported in the literature.

Extraction of the hemicellulose was accomplished by continuous percolation of alkali through a packed pulp bed. The bed was formed by swelling 1000 g. of pulp in 6 liters of 5% potassium hydroxide and packing this wet pulp in a 33-cm. Buchner funnel. Twelve liters of 5% potassium hydroxide followed by 18 liters of 10% sodium hydroxide plus 4% sodium borate were passed through the bed to accomplish the extraction. A total of 4000 g. of pulp was extracted by this procedure.

The extract was collected in 1-liter batches and tested for glucomannan content by the addition of 2.6M barium acetate solution to a 5-ml. aliquot. Virtually all of the precipitable glucomannan was contained in 8 liters (10th - 18th l.) of total extract.

Isolation of the glucomannan was accomplished by selective precipitation of the glucomannan-barium complex (35). The 8 liters of extract containing the major portion of the glucomannan from each 1000 g. pulp were treated with 665 ml. of 2.6M barium acetate. The resulting solution was 0.2M in barium ion which was sufficient to precipitate the glucomannan. The precipitate was filtered on a large Buchner funnel and washed with 10% sodium hydroxide. The complex was dissolved in 3 liters of water maintained at pH 5-6 by the addition of acetic acid. The barium complex was reprecipitated from this solution by the addition of 500 ml. of 7N sodium hydroxide. This complex was washed and dissolved in 3 liters of 1N acetic acid. The second precipitation-dispersion step was repeated for a total of three barium precipitations.

The purified glucomannan was precipitated from acidic solution by the addition of one volume of 95% ethanol. The precipitate was separated in the Sharples super centrifuge and stored as a wet paste at 0°C. until freeze dried.

Freeze drying was accomplished from a 3% water suspension. The product was a fine white powder. It showed no crystallinity by x-ray diffraction.

A total of 104 g. of purified glucomannan was prepared by the above procedure. Yield of the glucomannan was 2.6% of the pulp.

CHARACTERIZATION OF THE GLUCOMANNAN

SUGAR ANALYSIS

The glucomannan was analyzed for sugars content by the method of Saeman, et al. (34). The hydrolyzate contained mannose, glucose, and galactose only. The following yields were based on the weight of ash-free glucomannan.

	Anhydrosugar, %	Sugar Ratio
Mannan	74.4	3.25
Glucan	22.9	1.00
Galactan	1.2	0.05
Total	98.5	

Xylose and arabinose were apparently absent in the hydrolyzate since no evidence of these sugars could be found on chromatograms spotted with 3500 μg. of total sugars.

ASH ANALYSIS

Ash analyses were made by carbonizing oven-dried samples of the glucomannan below 300°C. and sulfating the charred residues with concentrated sulfuric

acid. Ignition temperature was 650°C. Total sulfated ash was 4.3%, and ash expressed as barium was 2.5%.

OPTICAL ROTATION

Optical rotation was determined at 25.0°C. with a Zeiss-Winkle polarimeter giving

$$[\alpha]_D^{25} = -32° \text{ (c 1, 2M sodium hydroxide)}$$

INTRINSIC VISCOSITIES

Intrinsic viscosities were determined at 25°C. in a Cannon 100/A4 dilution viscometer. Solvents used were 1M cupriethylenediamine and 2M sodium hydroxide plus 1M boric acid. Figure 1 is a plot of the reduced viscosities versus concentration. Intrinsic viscosities determined were:

$[\eta] = 0.34$ in 1M cupriethylenediamine

$[\eta] = 0.40$ in 2M NaOH plus 1M H_3BO_3

NUMBER AVERAGE D.P.

The number average degree of polymerization of the nitrated glucomannan was determined by osmotic pressure measurements.

Nitration of a one-gram sample of glucomannan was accomplished by the method described in the Institute laboratory manual for Courses A-125, A-126. The nitrated sample was dried from methanol under vacuum at 35°C; yield, 1.6574 g. The sample was stored under ethanol and placed at the disposal of the Cellulose Department.

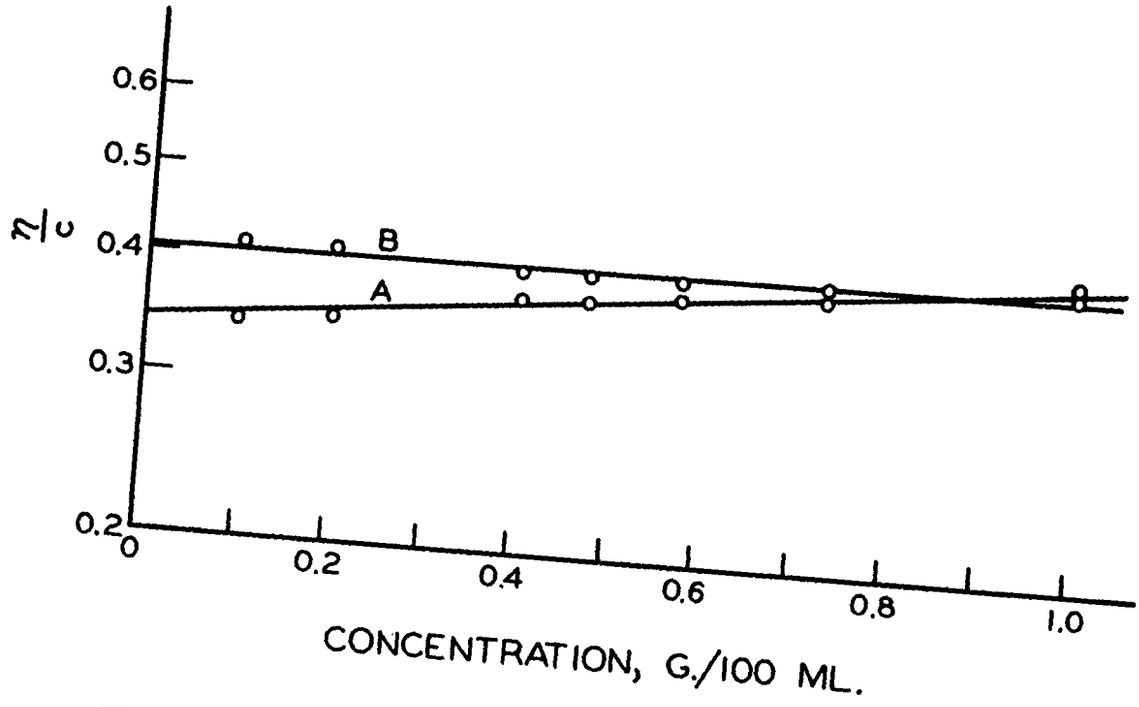


Figure 1. Log of Reduced Viscosity Versus Concentration.
Curve A - Solvent 1M Cupriethylenediamine.
Curve B - Solvent 2M NaOH plus 1M H_3BO_3.

A small amount (0.15 g.) of the nitrated material was sent to Geller Microanalytical Laboratories for nitrogen analysis. Nitrogen content was 12.96% which corresponds to a nitrate degree of substitution of 2.57.

Molecular weight was determined by Mr. Charles Schmitt of the Cellulose Department of the Institute. Osmotic pressure measurements were made at 30°C. in a Zimm-Meyerson osmometer equipped with Ultracella Allerfeinst membranes. The osmotic pressure/concentration quotient H/c was 1.33 at infinite dilution and corresponded to a number average molecular weight of 19,327 and a degree of polymerization of 70.

ELECTROPHORESIS OF THE GLUCOMANNAN

Boundary electrophoresis of the glucomannan was conducted in a Tiselius cell with the help of Dr. E. O. Dillingham.

Most electrophoresis runs on glucomannans have been made in borate buffers of high pH (10, 36-38). Use requirements of the Spinco Model H apparatus in the Biochemistry Department limited the maximum permissible pH to 9.0. For this reason, it was necessary to develop a new solvent system for the glucomannan. This was accomplished by dissolving 0.2 g. of glucomannan in 2 ml. of 2M sodium hydroxide-1M boric acid, then diluting with 15 ml. of water and 10 ml. of 0.6M boric acid. The resulting stable solution was 0.75% by weight glucomannan, 0.3M in borate ion, and had a pH of 8.2.

This buffer was shown to separate crude hemicellulose mixtures into multiple peaks (39). Electrophoresis was conducted at 30°C. with cell current 5-6 ma. Photographs of the positions of the boundaries were made with the Schlieren optical system at selected time intervals. Figure 2 was taken after 939 minutes of electrophoresis.

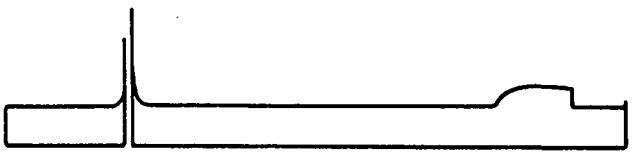


Figure 2. Electrophoretogram of the Glucomannan in Borate Buffer at pH 8.2

The single sharp peak indicates the glucomannan boundary. Apparently, the sample was composed of polymer chains which were electrophoretically homogeneous. The extreme sharpness of the peak, even after 15 hours, indicates very little diffusion which possibly means that most of the polymer chains were of substantial D.P.

A sample of solution was withdrawn from behind the boundary. A small amount of material was precipitated with 4 volumes of ethanol. This material and

a sample of the glucomannan as a control, recovered from the buffer solution were hydrolyzed and chromatographed in Solvent A. There was no apparent difference in mannose and glucose content in the two samples.

OXIDATION OF THE GLUCOMANNAN

DECOMPOSITION OF LEAD TETRAACETATE IN ACETIC ACID

The decay of lead tetraacetate in glacial acetic acid has been discussed by Detrick (29). This phenomenon was confirmed in this investigation. It was found that unsaturated solutions of lead tetraacetate in acetic acid lost about one half their oxidizing potential in the first 100 hours. It was found that the concentration of lead tetraacetate in saturated solutions over an excess of the salt remained remarkably stable at constant temperature, however. Table II illustrates this finding.

TABLE II

STABILITY OF LEAD TETRAACETATE IN SATURATED ACETIC ACID SOLUTION AT 25°C.

Time after Solution Preparation, hr.	Moles of Dissolved $Pb(OAc)_4$/l.
12	0.0882
42	0.0723
300	0.0725
3000	0.0724

The concentration of lead tetraacetate in this solution was sufficient to investigate a large range of oxidation conditions. An added benefit was that oxidation blanks prepared with saturated stock solutions had slower decay rates than those prepared from unsaturated stock solutions. Saturated stock solutions were used in all oxidation studies.

OXIDATION PROCEDURE

Several attempts were made to oxidize the glucomannan and ivory nut mannan A in heterogeneous suspensions. All of the pulp oxidation studies had been conducted by this procedure (26-29). The maximum oxidation obtained in any attempt corresponded to only 0.14 mole of lead tetraacetate consumed per sugar unit. This degree of oxidation was unsatisfactory for the requirements of this thesis.

Qualitative experiments indicated that homogeneous solutions of either mannan A or glucomannan in 80% acetic acid could be obtained by dissolving the polymers in 7N sodium hydroxide then slowly adding glacial acetic acid until 80% acid concentration was reached. The polymers precipitated after an extended period, but the freshly precipitated polymer was accessible to oxidation.

With this information at hand, a successful oxidation procedure was developed. The oxidation was studied under a variety of conditions by a series of small-scale reactions involving both the glucomannan and ivory nut mannan A. The procedure described in the following paragraphs was selected for the large-scale preparative oxidations. It is typical of all oxidation procedures used with the exception that the concentrations of certain components were varied as the studies required.

The reaction was carried out in 80% acetic acid. Glucomannan concentration was 0.5%, and the initial lead tetraacetate-to-sugar unit ratio was approximately 1.5. All operations were performed in a 25°C. constant temperature bath.

The glucomannan was prepared for oxidation by dissolving in 7N sodium hydroxide. Solution concentration was 10% glucomannan. After the polymer had dissolved completely, enough water was added to bring the water concentration for the final oxidation medium up to 20%.

The amount of saturated stock required for an initial oxidant-to-sugar unit ratio of 1.5 was determined by titration. A 2-ml. aliquot of stock solution was reduced with 15 ml. of a "stopping solution" (10 g. potassium iodide and 50 g. potassium acetate in 100 ml. water). The iodine which was liberated was titrated using Thyodene* as the indicator. This titration and the calculations required to determine oxidant consumed have been described in detail by Detrick (29).

The appropriate volume of lead tetraacetate stock solution was placed in a 3-liter, round-bottomed flask equipped with a sealed stirrer. The solution was diluted with enough glacial acetic acid so as to insure that the final oxidation solution contained 80% acetic acid. Reaction was initiated by pouring the alkaline solution of the glucomannan into the acetic acid solution with vigorous stirring. An oxidation blank was prepared by the same procedure and run concurrently with each oxidation sample.

The course of the reaction was followed by removing aliquots from the reaction flask at selected time intervals and titrating by the procedure described above. The difference in oxidant consumption between the oxidation blank and the sample was the amount of lead tetraacetate consumed by the glucomannan. By this procedure, values for a "consumption of oxidant versus time" curve were obtained.

At the end of the reaction, the precipitated glucomannan was separated by filtration and washed with 50% acetic acid. The filtrate and washings were combined, and the remaining lead tetraacetate was destroyed by the addition of aqueous oxalic acid. Precipitated lead oxalate was separated by filtration and washed with water.

*Thyodene is a commercial indicator for iodimetry produced by the Magnus Chemical Company, New York.

The material which had precipitated during the reaction was dissolved in water and combined with the filtrate. The clear solution was concentrated under vacuum on a rotary evaporator until the solution became turbid. The solution was then flooded with 10 volumes of absolute ethanol to precipitate the oxidized glucomannan. Drying was accomplished by solvent exchange through absolute ethanol and anhydrous ether.

The oxidized glucomannan was redissolved in water and passed through an ion-exchange column containing IR-120 resin to remove the metallic ash. The effluent from the column was concentrated to a thin sirup under vacuum, and the purified oxidized glucomannan was recovered by freeze drying.

DETERMINATION OF SUGAR RATIO IN OXIDIZED GLUCOMANNAN

A method was developed for rapidly determining the ratio of mannose to glucose in a polysaccharide hydrolyzate by direct measurement of the reflectance of the colored spots on a chromatogram. The method involved plotting the ratio of the Kubelka-Munk K/S values for the two sugars versus the ratio of the sugars in solution. Standard deviation about the regression line was 0.1, and the regression was independent of sugar concentration within the limits outlined in the experimental procedure. A detailed description of the method is included in Appendix I.

This method was used to follow the selectivity of the oxidation for the mannose units in the glucomannan during the course of the reaction. At selected times an aliquot containing 50-100 mg. of glucomannan was removed from the reaction flask. The oxidation was stopped by the addition of 2 ml. of ethylene glycol. The partially oxidized glucomannan was recovered and hydrolyzed by the method of Saeman, et al., (34). The hydrolyzate was concentrated, and the

mannose-to-glucose ratio was determined. By this procedure, values for an "unoxidized mannose-to-glucose ratio versus time" curve were obtained for the oxidation.

All mannose-to-glucose ratio determinations were made by this method unless noted otherwise.

TWO-STAGE OXIDATIONS

Several additional experiments were conducted in an attempt to explain the resistance of some of the mannose units to oxidation with lead tetraacetate. This involved the oxidation of glucomannan samples in two stages with various intermediate treatments. In all of these experiments the glucomannan was first oxidized by the normal procedure until the mannose-to-glucose ratio had been reduced to 1.5.

In one experiment the oxidized glucomannan was recovered from the initial oxidation solution, washed with glacial acetic acid and treated with fresh oxidant in a second step. No additional oxidation was noted and the mannose-to-glucose ratio remained unchanged.

The oxidized glucomannan as recovered from the first oxidation step contained 41% lead ash which was not removed by dialysis. This ash probably existed as a complex with the glucomannan, and, therefore, may have played a role in stabilizing the mannose units toward oxidation. In order to investigate this possibility lead was removed after the first oxidation step with IR-120 ion-exchange resin. The polymer was then subjected to a second oxidation step in which only 0.1 mole of lead tetraacetate per sugar unit was consumed after 3 hours time, and the mannose-to-glucose ratio was not changed. The lead content of the glucomannan after the second oxidation was only 9.4%.

A third two-stage oxidation investigation included the reduction of the dialdehyde units formed in the first oxidation with sodium borohydride. The reduction procedure used is described by Bishop and Cooper (10). With this intermediate treatment a large quantity of lead tetraacetate was consumed in the second oxidation step. The mannose-to-glucose ratio was reduced from 1.5 to 1.2 in one experiment and to 0.7 in another. Yields of oxidized products were only 7.5% of the glucomannan, however.

SELECTIVE DEGRADATION OF THE OXIDIZED POLYMERS

A series of small-scale experiments indicated that sulfurous acid smoothly hydrolyzed the oxidized glucomannan and ivory nut mannan, but did not degrade unoxidized glucomannan or cellobiose significantly. Therefore, this reagent was used to selectively degrade the polymers in an attempt to preserve, nearly intact, the unoxidized portions of the polymers.

Sulfurous acid was prepared by saturating distilled water with sulfur dioxide at 25°C. One hundred ml. of sulfurous acid and 2.0 g. oxidized glucomannan were placed in a 200-ml. round-bottomed glass pressure vessel. The polymer dissolved rapidly, forming a pale yellow solution. The vessel was capped tightly and placed in a 100°C. temperature bath for 3.5 hours.

After hydrolysis most of the sulfur dioxide was removed under vacuum. The remainder of the sulfite and sulfate ions were removed by precipitation with barium hydroxide, and excess barium was removed by balancing with sulfuric acid. The clear solution was concentrated to 10 ml. on a rotary evaporator. Benzoic acid, 10 mg., was added as a preservative, and the sirup was stored at 0°C. until used.

SEPARATION OF HYDROLYSIS PRODUCTS

PAPER CHROMATOGRAPHY

Small portions of the sulfurous acid hydrolyzate were investigated by paper chromatography in Solvents A and C. Several known compounds which were expected in the hydrolyzate were run concurrently. Table III presents the information obtained from this qualitative investigation. The symbols R_f and R_c refer to the mobility of the spot with respect to the solvent front and to known cellobiose, respectively.

TABLE III

PAPER CHROMATOGRAPHY OF THE SULFUROUS ACID HYDROLYZATE OF OXIDIZED GLUCOMANNAN AND KNOWN REFERENCE COMPOUNDS

	Solvent A		Solvent C
	R_f	R_c	R_c
Unknown spots			
A (light)	0.79		
B (light)	0.60		
M (heavy)	0.23		
G (heavy)	0.18		
C (light)		3.00	2.98
D (medium)		1.85	1.54
E (medium)		1.00	1.00
F (faint)		0.58	0.66
Heavy streak		0-0.6	0-0.8
Known spots			
glyoxal	0.7-0.9		
erythrose	0.65		
mannose	0.23		
glucose	0.18		
glucopyranosylmannose		1.90	1.42
mannobiose		1.05	1.21
cellobiose		1.00	1.00
mannopyranosylglucose		0.57	0.85

The information in Table III indicates that in addition to mannose, glucose, and the oxidation fragments, three of the four possible 1-4 linked disaccharides are probably present. Mannobiose appears to be absent. So do

any tri- or tetrasaccharides. Spot C and the heavy streak do not correspond to any known compounds. Mannobiose was apparently also absent from the sulfurous acid hydrolyzate of oxidized ivory nut mannan.

QUANTITATIVE SEPARATION

Glucose and mannose in the hydrolyzate were determined photometrically by the Saeman procedure (34). This procedure was modified slightly by the omission of the unneeded sulfuric acid hydrolysis. The analysis indicated that the hydrolyzate contained 16.1% mannose and 15.0% glucose. Glyoxal and erythrose were not determined quantitatively because they were known products of the oxidation and their determination was not considered significant.

The disaccharides were separated by cellulose column chromatography. A 5 by 48 cm. column was packed with Whatman cellulose powder by the general sedimentation method described by Green (40). The powder was suspended in Solvent A for column preparation. Since this solvent was used for elution, solvent exchange was omitted in the preparation step.

The entire hydrolyzate was adsorbed on 5 g. of cellulose powder. This powder was then placed on the column with a minimum of solvent and allowed to settle into a uniform layer. The monosaccharides were eluted with Solvent A. Elution of the disaccharides was speeded by changing the solvent ratio to ethyl acetate–pyridine–water, 6:3:3.

After the elution of the monosaccharides, fractions containing 100 ml. each were collected with a Technicon automatic fraction cutter. The contents of these bottles were checked by paper chromatography, and those containing the same components were combined. The combined fractions were concentrated on a rotary evaporator and dried over calcium chloride under vacuum.

Table IV is a compilation of the data obtained on the crude fractions taken from the column. The information under chromatographic identification represents qualitative identification of the major components of the fraction by paper chromatography in Solvents A and C.

TABLE IV

CELLULOSE COLUMN CHROMATOGRAPHY OF SULFUROUS ACID HYDROLYZATE

Fraction	Crude Weight, g.	Chromatographic Identification
1	0.0545	Unknown C
2	0.0522	C and glucosidomannose
3	0.1048	Glucopyranosylmannose
4	0.0815	Glucopyranosylmannose and cellobiose
5	0.1188	Cellobiose
6	0.1123	Small amounts of cellobiose
7	0.7495	Heavy streak to base line

These results are in good agreement with those obtained from qualitative paper chromatography of the whole hydrolyzate. All fractions contained small amounts of glucose and mannose and considerable material which remained at or near the base line on paper.

INVESTIGATION OF FRACTIONS

Fractions 2, 4, and 6 were overlapping mixtures and were not investigated further.

The major component of Fraction 1 was unknown C. This material had a higher mobility in Solvents A and C than any known disaccharide. It could not be hydrolyzed by 3% sulfuric acid at 100°C. which cleaves known β-1-4 linkages. However, severe hydrolysis with sulfuric acid at 120°C. destroyed the unknown but did not liberate either glucose or mannose. In fact, much of the material remaining after hydrolysis exhibited identical chromatographic behavior to Fraction 7.

Fraction 7 was a dark brown material which formed a continuous streak on paper chromatograms. The leading edge of this streak had a mobility with respect to cellobiose of about 0.6 in Solvent A and 0.8 in Solvent C. Extended hydrolysis with sulfuric acid at 100°C. and at 120°C. and with sulfurous acid at 100°C. did not release significant amounts of mannose or glucose from this material. This unknown did not appear to be composed of larger oligosaccharides or of unhydrolyzed polymer.

The absence of recoverable hexose units in Fractions 1 and 7 suggested the possibility that these materials were formed by glyoxal or erythrose condensation during the hydrolysis with sulfurous acid. Therefore, glyoxal, erythrose, and a mixture of the two were treated with sulfurous acid under the conditions used to hydrolyze the oxidized glucomannan. The hydrolyzates were chromatographed in Solvents A and C. Glyoxal normally streaks badly, but the great majority of the material is concentrated between R_f 0.7 and 0.9. After hydrolysis the samples containing glyoxal exhibited two heavy streaks on paper in the mobility range of Fractions 1 and 7. Only a faint spot was noted near the solvent front. These chromatograms also indicated that erythrose was lost during heating with sulfurous acid.

Fractions 1 and 7 were not composed of sugar units. Therefore, they were of little importance to the major points of this thesis, and were not investigated further.

The major components of Fraction 3 had the same chromatographic mobility as 4-O-β-D-glucopyranosylmannose in Solvents A and C. However, considerable amounts of this fraction formed a dark streak near the base line. For this reason, the fraction was purified by quantitative paper chromatography.

The fraction was placed on a 22 by 24 in. Whatman no. 3 MM sheet in three 5-inch streaks separated by one-half inch guide spots. The chromatogram was eluted with Solvent A for 48 hours. After elution, the carrier strips were cut from the sheet, and the guide spots were developed with aniline–monochloroacetic acid developer. The portions of the carrier strips which contained the disaccharide were cut out and eluted with water.

The purified disaccharide was recovered as a tan sirup, and the following information about this material was obtained.

1. Yield, 0.0202 g.
2. Optical rotation $[\alpha]_D^{23}$ = + 9.8° (c, 1.01 in water).
3. Hydrolysis produced mannose and glucose in 1:1 ratio.
4. Chromatographically identical to glucopyranosylmannose.

Unfortunately, attempts to crystallize the disaccharide were unsuccessful. Therefore, the octaacetate derivative was prepared by the procedure described by Merler (41). The derivative was recrystallized from 95% ethanol and compared with an authentic sample of O-octaacetyl-4-O-β-D-glucopyranosyl-α-D-mannose.

Glucopyranosylmannose α-octaacetate m.p., °C.	199-200[a]
Prepared derivative m.p., °C.	198-200
Mixed m.p., °C.	198-200

[a]Melting points uncorrected.

The infrared spectra of the two acetates were also identical.

From these data, it was concluded that the major component of Fraction 3 was 4-O-β-D-glucopyranosylmannose.

The principal component of Fraction 5 was a spot which had the same mobility as cellobiose in Solvents A and C. However, it was also contaminated with considerable impurities. Therefore, it was purified by the paper chromatographic procedure described above.

Three subfractions were eluted from the paper. The first, Fraction 5-1, contained mostly colored impurities. The principal spot had a R_c of 0.6 in Solvent A and 0.74 in Solvent C. Hydrolysis of the subfraction afforded chromatographic spots for mannose and glucose in approximately equal amounts.

These data indicate that small amounts of mannopyranosylglucose were probably present in this subfraction; yield of impure material 0.0177 g.

The second subfraction, 5-2, was isolated from the leading edge of the major component of Fraction 5. Chromatographic analysis of this material showed that it had an R_c of 1.0 in Solvent A and an elongated R_c 1.0-1.1 in Solvent C. The leading edge of the elongated spot moved slightly more slowly than did mannobiose in the Solvent C system. Hydrolysis of this subfraction afforded mostly glucose. A small amount of mannose was also found indicating that mannobiose also may have been present in trace amounts. The indications were that this subfraction was composed mainly of cellobiose; yield, 0.0120 g.

The largest subfraction, 5-3, was concentrated to an amber sirup. The following information was obtained on this material.

1. Yield, 0.0265 g.
2. Optical rotation, $[\alpha]_D^{23}$ + 30.8° (c, 0.88 in water).
3. Hydrolysis produced glucose only.
4. Chromatographically identical to cellobiose.

Fraction 5-3 was crystallized from aqueous acetic acid, washed with acetone and dried under vacuum. The melting point was compared with an authentic sample of cellobiose.

Cellobiose m.p., °C.	231-233[a]
Fraction 6-3 crystals m.p., °C.	231-233
Mixed m.p., °C.	230.5-233

[a]Melting points uncorrected.

The infrared spectrum of the crystals was identical to the spectrum of cellobiose. Therefore, it was concluded that Fraction 5-3 and most of the Fraction 5-2 were composed of cellobiose.

DISCUSSION OF RESULTS AND CONCLUSIONS

GLUCOMANNAN ISOLATION

White spruce was chosen as a source of glucomannan because spruce glucomannans have been studied extensively, and white spruce glucomannan has been particularly well characterized (12).

The glucomannan was extracted from a sulfite pulp instead of from a holocellulose because (a) a pulp could be prepared more easily, and (b) the acid cook would remove most of the galactose units from the glucomannan. Since these units existed as terminal side chains, they were of no importance to the oxidation study.

Although normal acid sulfite cooks remove most of the glucomannan, Annergren and Rydholm (31-33) found that the glucomannans of softwoods became more resistant to hydrolysis during sulfite or bisulfite pulping if the cook was preceded by a long penetration time or by a precook at higher pH. Best results were obtained when this precook was slightly alkaline. In addition, their data on α-cellulose yields and on the compositions of alkali extracts showed that most of the stabilized glucomannan was extractable with alkali.

The two-stage cook employed in this thesis was based on the above work. The pulp prepared had a mannan content of 12.7%, which was much higher than the mannose content (3-5%) normally found in conventional sulfite pulps.

Identical alkaline extractions were conducted on two 100-g. samples of the pulp. One sample had been pre-extracted with alcohol–benzene and cold water, the other had not. Each sample was subjected to a 5% potassium hydroxide extraction followed by an extraction with 10% sodium hydroxide containing 4% sodium borate. The results are shown in Table V.

TABLE V

EFFECT OF ALCOHOL—BENZENE PRE-EXTRACTION ON THE EXTRACTION OF HEMICELLULOSE WITH ALKALI

	Pulp Pre-extracted With Alcohol—Benzene		Pulp With No Pre-extraction	
	KOH	NaOH	KOH	NaOH
Hemicellulose yield, %	3.8	12.2	3.4	14.0
Mannan, %	24.5	53.2	24.7	53.1
Glucan, %	10.8	17.8	10.9	17.4
Xylan, %	49.1	17.2	44.3	19.1

The data in Table V indicate that pre-extraction with alcohol—benzene had little effect on the quality of extractable hemicellulose. For this reason, pre-extraction was omitted in the large-scale hemicellulose isolations.

A comparison of the potassium hydroxide and sodium hydroxide-extractable hemicelluloses indicated that prior extraction of the pulp with potassium hydroxide served a useful purpose removing large amounts of the xylan component. Although some glucomannan was removed by this extraction, the reduction in xylan content made purification of the sodium hydroxide-extractable glucomannan more efficient. Therefore, a 5% potassium hydroxide pre-extraction was included in large-scale isolations of the glucomannan.

The continuous extraction described in the experimental procedure was designed to dissolve the glucomannan in a minimum volume of alkali. Percolation of the alkali through the pulp bed concentrated the glucomannan near the sodium hydroxide solvent front instead of dispersing it evenly throughout the total volume. This made it possible to collect the crude glucomannan fraction from 4000 g. of pulp in 32 liters of alkali, whereas more than 100 liters would have

been required with conventional batch extraction procedures. Similarly, the amount of potassium hydroxide required for xylan removal was also lowered.

The glucomannan purification procedure involved three major modifications of the isolation procedure developed by Meier (35).

First, 2.6M barium acetate instead of saturated barium hydroxide was added to effect precipitation. Barium acetate proved to be as efficient a precipitating agent in excess alkali as barium hydroxide, and its greater solubility allowed solution volumes to be held to a minimum.

Second, the glucomannan was selectively precipitated directly from the alkali-borate solution. Previous isolation procedures have included complete precipitation of the hemicellulose with large volumes of ethanol and redispersion in alkali alone prior to any selective precipitation. This time-consuming step was found to be unnecessary.

The direct precipitation was responsible for the third modification of Meier's procedure. Excess barium acetate was required to precipitate the glucomannan in the presence of borate ion. In fact, the initial complex was so rich in barium that subsequent precipitation steps were performed by pH adjustment alone. This factor may possibly have enhanced the glucomannan selectivity of the second and third precipitations. The barium ions which were released from the glucomannan complex upon acidification were uniformly dispersed throughout the solution instead of being concentrated at the point of addition. This lower uniform concentration should have minimized the coprecipitation of xylan contaminant. In any event, only three precipitations of the barium complex were required to isolate a glucomannan completely free of xylose units. Meier reported small amounts of xylan even after four precipitations.

Table VI lists the properties of several spruce glucomannans. The data in this table indicate that the glucomannan isolated in this work was similar to others reported in the literature. The properties of this polysaccharide compare remarkably well with those of the one isolated from white spruce holocellulose. Therefore, information obtained about the two polymers can be combined in order to obtain a more complete structure for the glucomannan.

The data in this table led to the conclusion that fairly representative glucomannan fractions can be isolated in large quantities by the above simplified procedure. Therefore, this isolation procedure should be useful in future studies requiring large supplies of purified polysaccharide.

In addition, the results of boundary electrophoresis experiments suggest that the glucomannan was composed of polymer chains similar in mannose unit content.

An important adjunct to the electrophoresis procedure was the development of a glucomannan-borate solution at pH 8.2. The glucomannan solution was stable even after standing for weeks. The lower pH of this solvent probably causes less degradation to the polymer than does a normal alkali-borate solvent. Therefore, it should find many applications in future physicochemical studies.

OXIDATION OF THE GLUCOMANNAN

Several studies of the lead tetraacetate oxidation of wood pulps suspended in acetic acid have been made (26-29). Perlin also attempted to oxidize xylan and glucan polysaccharides in the same manner (25). In all of these experiments only a small amount of oxidant was consumed.

TABLE VI

PROPERTIES OF SPRUCE GLUCOMANNANS

Source	Mannose-to-Glucose Ratio	Optical Rotation, degrees[a]	Intrinsic Viscosity[b]	Degree of Polymerization $\overline{DP}_n$	Degree of Polymerization $\overline{DP}_w$	Reference
White spruce two-stage pulp	3.2	-32	0.34	70	---	This Work
Holocellulose	3.1	-34	0.24	108	180	(12)
Sitka spruce holocellulose	2.5	-33	---	49	---	(4)
Norwegian spruce sulfite pulp	3.5	-39	---	28-46	---	(42)
Holocellulose	3.6	---	---	71-117	60-130	(3)
Black spruce sulfite pulp	3.2	---	0.25	---	148	(43)
Holocellulose	3.4-3.8	---	0.38-0.43	---	225-254	(43)

[a]Optical rotation in 1N NaOH.

[b]Intrinsic viscosity in 1M cupriethylenediamine.

This was expected for two reasons. First, the accessibility in any heterogeneous system plays a role in the degree and speed of chemical reaction. Secondly, the major portion of the vic diols in all of the polysaccharides studied were in the trans conformation. Perlin (25) has shown that these groups, when they occur on central residues of 1,4-linked polymers, resist oxidation with lead tetraacetate. No instance was found in the literature where polysaccharides with a large number of cis-glycol groups were oxidized. Nevertheless, it was assumed that such a polymer would be more easily oxidized, even in a heterogeneous medium, than the ones studied previously.

Attempts were made to oxidize samples of solvent-dried glucomannan and ivory nut mannan A, and freeze-dried glucomannan in acetic acid suspension. The procedure used was similar to that of Detrick (29). In some samples potassium acetate was added to catalyze the reaction (25). However, the maximum oxidation obtained in any attempt consumed only 0.14 mole of oxidant per mole of anhydrohexose. This degree of oxidation was unsatisfactory. Apparently, even in an amorphous powder the majority of the cis-glycols were inaccessible to oxidation by lead tetraacetate. These findings indicate that accessibility factors other than crystallinity, as measured by Detrick (29), inhibit the oxidation of polysaccharides in acetic acid suspension.

Failure to obtain significant amounts of oxidation with heterogeneous suspensions necessitated development of a new oxidation procedure. Baer and co-workers (44) showed that glycosides could be oxidized with lead tetraacetate in aqueous acetic acid systems, and Perlin (24) noted that sodium acetate was an effective catalyst for the lead tetraacetate oxidation of oligosaccharides. This information led to the glucomannan oxidation procedure developed in this thesis.

The glucomannan was dissolved in aqueous sodium hydroxide. Oxidation was initiated by pouring the alkaline solution into the acetic acid solution of lead tetraacetate. A catalyzed oxidation in aqueous acetic acid was a direct result of these operations. Although some of the glucomannan precipitated on acidification, it remained accessible to the oxidant. After oxidation, the mannose and glucose contents of the fraction which precipitated were identical with those of the fraction which remained in solution.

The oxidation was studied under a variety of conditions. The effects of (a) polymer concentration, (b) the molar ratio of initial oxidant to sugar unit, and (c) water concentration on the consumption of lead tetraacetate and on the mannose-to-glucose ratio in the oxidized polymer were determined. The purpose of these studies was to obtain conditions which would lead to maximum oxidation of the mannose units and minimum oxidation of the glucose units.

Figure 3 shows the effect of water concentration on the consumption of lead tetraacetate. Addition of water to the reaction medium increased the oxidation rate dramatically.

This same effect was noted in the oxidations of simple sugars (44, 45). Criegee and Buchner (46) attributed it to the formation of "nascent" lead dioxide which was a more effective oxidant than lead tetraacetate itself. Cordner and Pausacker (47) considered the change in dielectric constant of the medium to be a more important reason for the enhancing effect. In either case, the water probably promotes the formation of the cyclic intermediate (page 5) required for glycol cleavage.

In the reaction containing 20% water, the residual mannose-to-glucose ratio was reduced from 3.2 to 1.5 in 60 minutes. This ratio was 1.9 after 1440

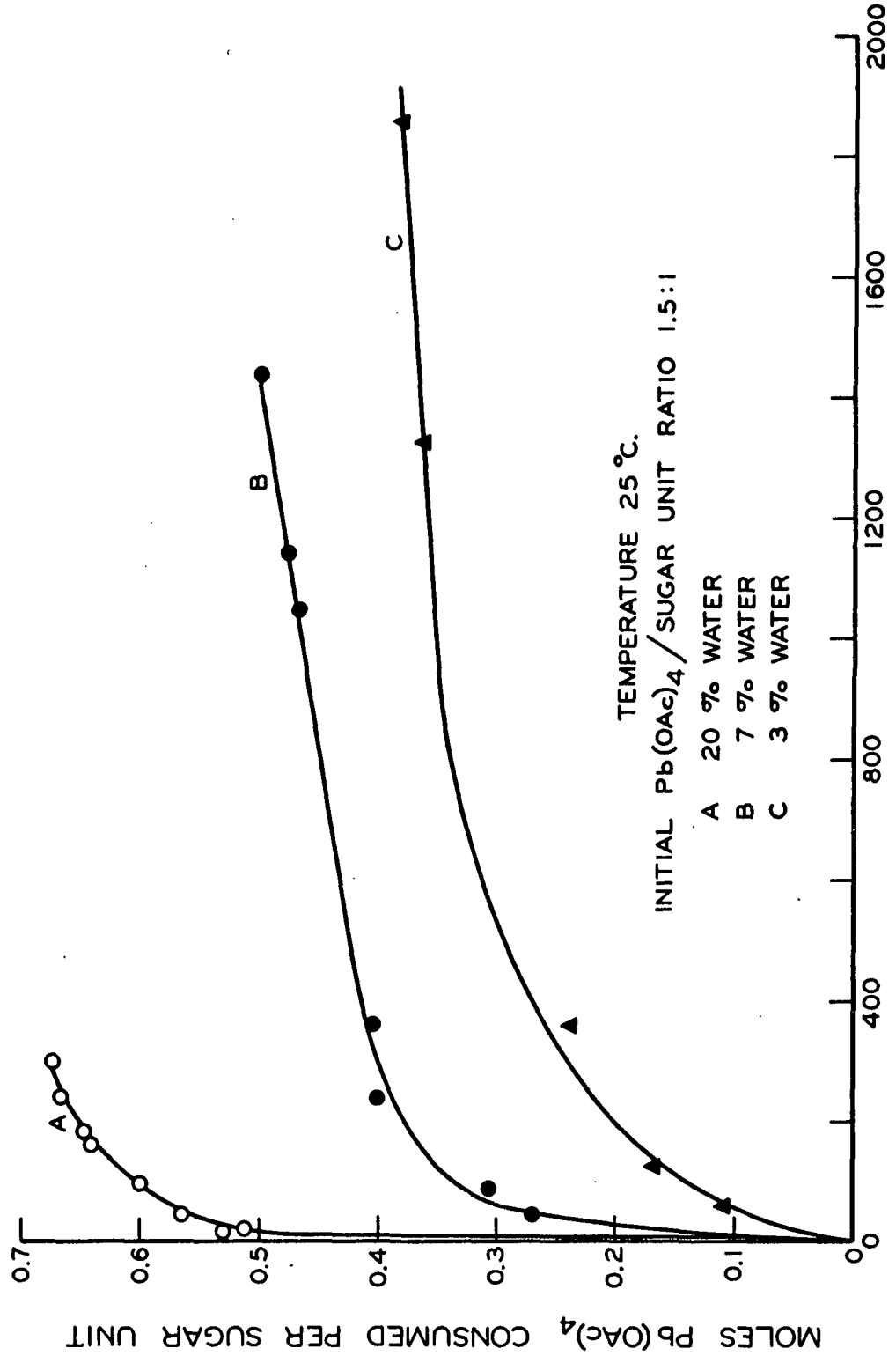


Figure 3. Effect of Water Concentration on the Consumption of Lead Tetraacetate by the Glucomannan.

minutes in the reaction containing 7% water and 2.7 after 1850 minutes in the reaction containing 3% water. The reactions appeared to be less specific at the lower water concentrations because in both of these reactions the amounts of oxidant consumed were greater than could be accounted for by the decrease in mannose-to-glucose ratio. In any case, the reaction containing 20% water was the only one which approached completion in a reasonable period of time. Therefore, this concentration was selected for further oxidation studies.

Figure 4 shows the effect of glucomannan concentration and the initial molar ratio of lead tetraacetate to monomer sugar unit on the consumption of oxidant with time. Figure 5 shows the effects of these reaction variables on the mannose-to-glucose ratio in the unoxidized portion of the polymer.

Curves A vs. B and C vs. D in Fig. 4 compare the effects of polymer concentration on the rate of oxidant consumption. The rate increases with polymer concentration in both cases, but the increase is hardly significant at the higher initial oxidant level. Curves B vs. C compare the effects of different initial molar ratios of oxidant to sugar unit on the rate of lead tetraacetate consumption. The increase in rate between these two reactions was quite dramatic. Comparison of all of the curves in Fig. 4 led to the conclusion that an increase in either of these reaction variables increased the rate of oxidant consumption, but the initial molar ratio of oxidant to sugar unit was the dominant variable.

The most surprising result of the oxidation studies is shown in Fig. 5. This figure is a plot of the decrease in mannose-to-glucose ratio in the glucomannan with time of oxidation. The curve is a composite of all of the reactions shown in Fig. 4. Although there were large differences in oxidant consumption in these reactions, the degree of selectivity for the mannose units was the same under all conditions. In each case the ratio was reduced from 3.25 to 1.5 and

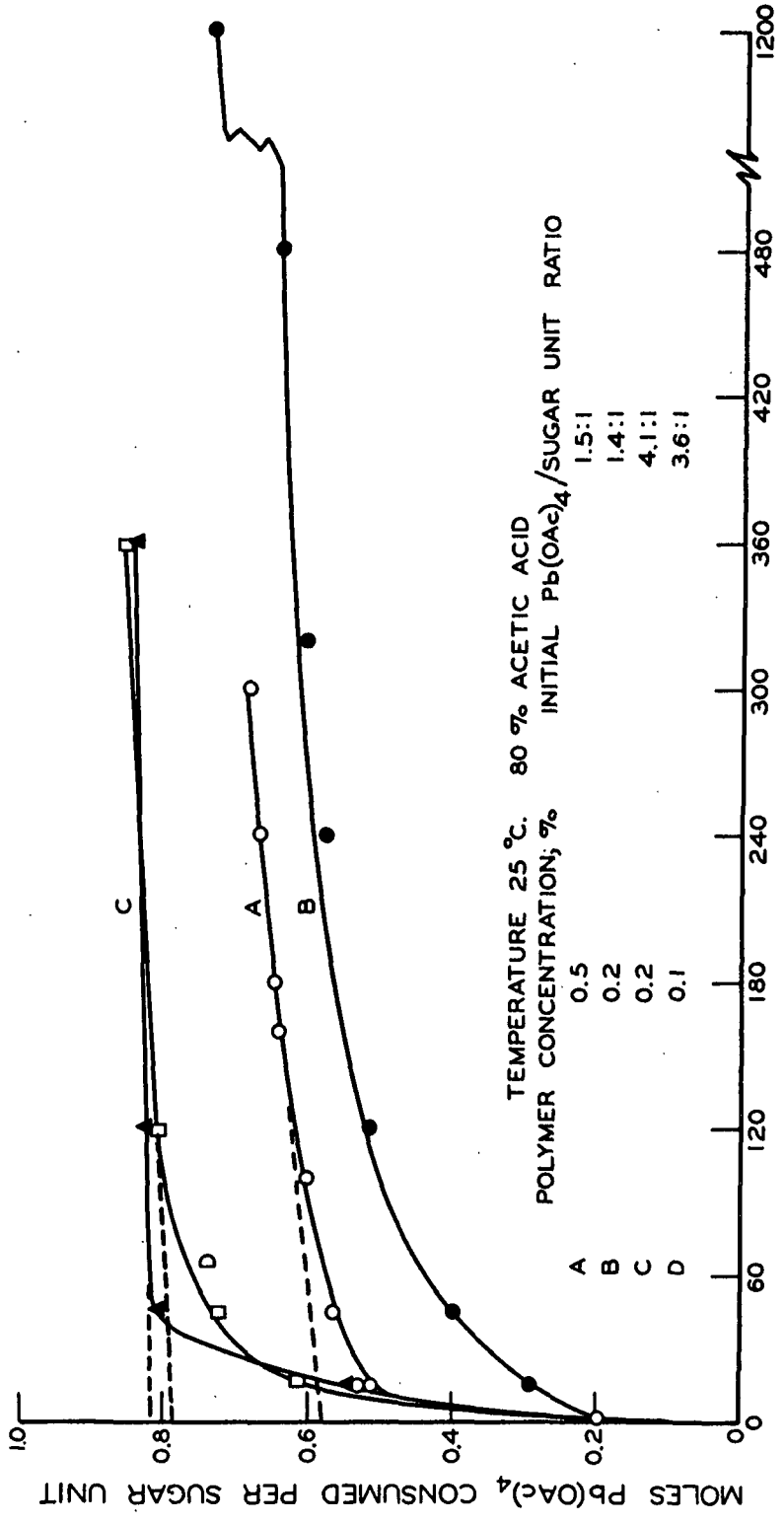


Figure 4. Effect of Polymer Concentration and Initial Molar Ratio of Oxidant to Sugar Unit on the Consumption of Lead Tetraacetate by the Glucomannan

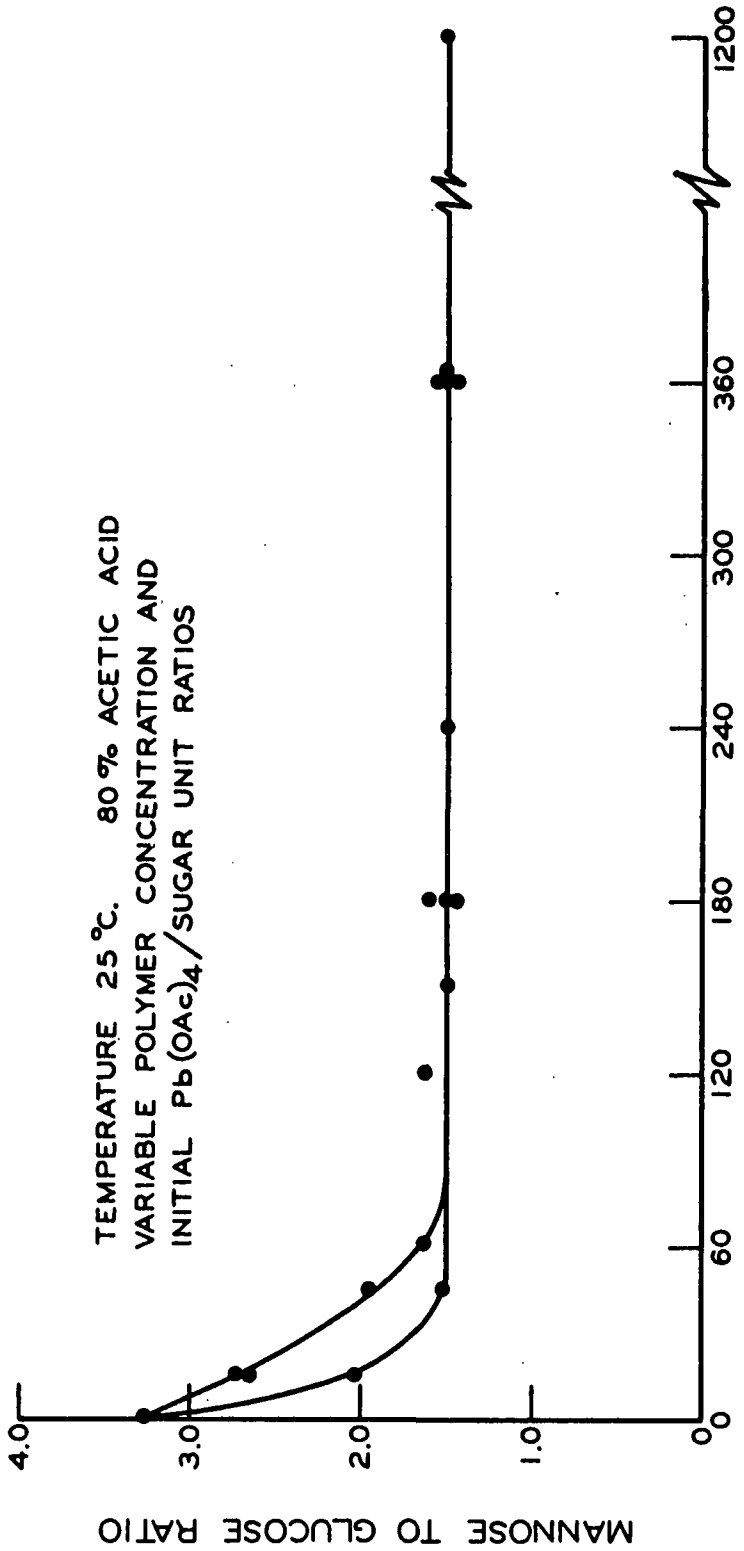


Figure 5. Decrease in Mannose to Glucose Ratio in the Glucomannan with Oxidation Time.

then was stable even though the oxidant consumption continued to rise. Therefore, the excess consumption was probably due to nonselective oxidation of both mannose and glucose units and/or to nonspecific oxidation reactions. These reactions may have been similar to those discussed by Bobbit (48) for the periodate oxidation.

The fact that the oxidation curves in Fig. 4 (consumption versus time) have a noticeable break after a certain time of reaction indicates that an "initial" oxidation had taken place (49, 50). The break in the curve in Fig. 5 shows that this "initial" reaction is concerned mostly with the selective oxidation of mannose units. Therefore, it should be possible to obtain a measure of oxidant consumed in this "initial" reaction by extrapolation of the curves after the break to zero time (29). Table VII lists these extrapolated values.

TABLE VII

INITIAL CONSUMPTION OF LEAD TETRAACETATE BY GLUCOMANNAN IN 80% ACETIC ACID AT 25°C.

Curve	Glucomannan Concentration, %	Initial Oxidant to Sugar Unit Ratio	"Initial" Consumption, moles oxidant/sugar unit
A	0.5	1.5	0.58
B	0.2	1.4	0.58
C	0.2	4.1	0.81
D	0.1	3.6	0.79

The oxidant consumption curves do not extrapolate to the same value, although in all of the reactions the mannose-to-glucose ratio was reduced from 3.2 to 1.5.

The value of this stable ratio indicates that at least 54% of the non-terminal mannose units were selectively oxidized even if there were no destruction of nonterminal glucose units. Sample calculations were made in order to predict

the theoretical oxidant consumption for such a minimum oxidation. These calculations were based on the following considerations.

1. Number average D.P. of 70.
2. No oxidation of internal glucose units (25).
3. Rapid oxidation of all end groups regardless of glycol configuration (24).
4. Possibility of rapid hydrolysis of formate esters formed by the oxidation of reducing end groups (24).
5. Rapid oxidation of formic acid to carbon dioxide with consumption of additional lead tetraacetate (24).
6. All galactose units present as nonreducing end groups of branches.

The results of these calculations are shown in Table VIII. These calculated values are lower than the extrapolated values in Table VII. However, the extrapolated values for the oxidations performed with initial oxidant molar ratios of 1.5 are much closer to the theoretical values than are those of the oxidations performed with the higher initial oxidant ratios. In addition, the slopes of Curves A and B (Fig. 4) after the "break" are greater than the slopes of Curves C and D. This probably indicates that nonselective oxidations occur concurrently with the selective reactions to the point that the concept of extrapolation to zero time had no validity.

Thus, it was concluded that, unlike periodate, the measurement of the consumption of lead tetraacetate by a glucomannan is not useful in predicting the structure of the polymer. Conditions necessary for the selective oxidation also promote nonselective oxidations which lead to erroneously high values of oxidant consumption. Nevertheless, this conclusion did not preclude the use of lead tetraacetate as a selective oxidant for the glucomannan. Reaction conditions had very little effect on the degree of selectivity, however.

TABLE VIII

THEORETICAL MINIMUM CONSUMPTION OF LEAD TETRAACETATE BY INITIAL SELECTIVE OXIDATION

Source of Oxidation	Moles of Lead Tetraacetate Consumed Per Sugar Unit	
	Without Formate Ester Hydrolysis	With Formate Ester Hydrolysis
Internal Mannose Units	0.404	0.404
Reducing End Groups	0.031	0.071
Nonreducing End Groups	0.072	0.072
Total	0.507	0.547

The reaction conditions represented by Curve A, Fig. 4, were chosen for the large-scale oxidations. These conditions promoted the selective oxidation in a reasonable length of time, and apparently minimized the nonspecific oxidations. A total of 25 g. of glucomannan was oxidized in three batches. The oxidations were followed by measuring the consumption of lead tetraacetate with time, and the reactions were allowed to proceed beyond the "break" in the oxidation curve. At termination the amounts of oxidant consumed in the three batches were 0.64, 0.64, and 0.63 moles of lead tetraacetate per sugar unit. Combined recovery of the oxidized polymer represented 65.5% of the original glucomannan.

Figures 7, 8, and 9 in the Appendix show the reaction curves obtained in the large-scale oxidations, and also the reproducibility of the oxidation curves shown in Fig. 4 and 5.

SELECTIVE DEGRADATION OF THE OXIDIZED GLUCOMANNAN

The classical means of degrading a periodate or lead tetraacetate oxidized polysaccharide selectively is the Barry degradation (51). In this

reaction phenylhydrazine cleaves the glycosidic linkages of the oxidized units but does not attack the linkages between unoxidized residues. However, the use of phenylhydrazine has the serious drawback that phenylhydrazones are formed both from the degradation products and from the unoxidized saccharides. Furthermore, the removal of the phenylhydrazine to recover the sugars results in considerable losses. Hence, another type of selective degradation was used.

Moyer and Isbell (52), studying periodate-oxidized dextrans, found that although the dialdehyde groups were fairly resistant to hydrolysis with mineral acids they were rapidly degraded with sulfurous acid. Mild hydrolysis with this acid led to almost quantitative recovery of oxidation products. In contrast, hydrolysis with hydrochloric acid or sulfuric acid led to product recoveries of only 50-65%.

Ewald and Perlin (53) compared the action of this reagent on a periodate-oxidized araboxylan with that of phenylhydrazine. In parallel experiments, these workers found chromatographic evidence that hydrolysis with sulfurous acid gave the same products as the Barry degradation procedure.

Therefore, sulfurous acid was used to degrade the oxidized glucomannan. Several small-scale hydrolyses were made in order to confirm the selectivity of this reagent for the oxidized portions of the polymer. The oxidized polymer was hydrolyzed to mono- and disaccharides; under the same conditions the unoxidized glucomannan released only insignificant amounts of oligosaccharides, and cellobiose was not attacked significantly. From these experiments it was concluded that a mild hydrolysis with sulfurous acid attacked the oxidized portions of the glucomannan preferentially and left the glycosidic linkages between unoxidized sugar units largely untouched.

These conclusions were supported by the results of a small-scale Barry degradation of the oxidized glucomannan. Although the products of this reaction were not identified, they were soluble in absolute ethanol and moved rapidly on a paper chromatogram eluted with toluene—ethanol—water, 270:90:1 (53). It was assumed from these results that the polymer was oxidized uniformly and that unoxidized fragments with D.P.'s greater than 2 or 3 did not survive the oxidation.

SEPARATION OF HYDROLYSIS PRODUCTS

Qualitative paper chromatography of the sulfurous acid hydrolyzate indicated the presence of mono- and disaccharides, only (Table III, page 23). No evidence of oligosaccharides with higher D.P.'s was found. In the absence of higher oligosaccharides, a cellulose column was considered to be more efficient than charcoal for the separation of the mixed disaccharides. Therefore, this medium was used for the large-scale separation of the sugars.

Table IX lists the yields of the anhydrosugar units determined in the sulfurous acid hydrolyzate. Anhydromannose, glucose, and galactose contents of the unoxidized and the oxidized glucomannan obtained by total hydrolysis with sulfuric acid are also shown in this table for the purposes of discussion.

A comparison of the sugar contents of the two total hydrolyzates indicates that although the oxidation was partially selective for the mannose units, glucose was also oxidized significantly. This fact supports the conclusion that the oxidation is not completely selective.

The absence of galactose in the oxidized polymer is in accord with the evidence in the literature that this sugar is present only as terminal units in the glucomannans.

TABLE IX

ANHYDROSUGAR UNIT CONTENT OF GLUCOMANNAN HYDROLYZATES

Anhydrosugar Unit	Unoxidized Glucomannan[b] Total Hydrolysis Sulfuric Acid	Oxidized Glucomannan[c] Total Hydrolysis Sulfuric Acid	Oxidized Glucomannan[c] Selective Hydrolysis Sulfurous Acid
Mannose, %	74.4	24.4	14.5
Glucose, %	22.9	16.5	13.5
Galactose, %	1.2	Nil	Nil
Cellobiose, %	---	---	1.8 (2.5)[a]
Glucopyranosylmannose, %	---	---	1.0 (1.6)[a]
Mannopyranosylglucose, %	---	---	Trace
Mannobiose, %	---	---	Trace (?)

[a]Figures in parentheses are the estimated total amount in the hydrolyzate.

[b]Per cent based on original glucomannan.

[c]Per cent based on oxidized glucomannan.

There was a significant difference in mannose content between the total acid hydrolyzate and the selective acid hydrolyzate of the oxidized glucomannan, but this difference was not investigated and no explanation is at hand.

On the other hand, the recovery of glucose from the selective hydrolysis was quantitative. Based on the estimated total yields of cellobiose and glucopyranosylmannose, 16.9% of the sulfurous acid hydrolyzate was recovered as anhydroglucose versus 16.5% recovered from the total acid hydrolyzate. Total yields of the disaccharides were determined by adding the estimated amount of each disaccharide in the overlapping fractions (Table IV, page 25) to the quantitative yield of the pure fractions.

Of the four possible β-1-4 linked disaccharides, only cellobiose and glucopyranosylmannose were identified unequivocally from the sulfurous acid hydrolyzate. There was good chromatographic evidence for the presence of trace amounts of mannopyranosylglucose, but the presence of even traces of mannobiose was questionable. The only evidence for the presence of this disaccharide was a trace of mannose which was detected chromatographically in the hydrolyzate of cellobiose fraction 5-1 (page 28). The significance of these findings is discussed in the succeeding sections.

NATURE OF THE GLUCOSE LINKAGES

The isolation of cellobiose from the sulfurous acid hydrolyzate is prima facie evidence for the existence of glucose-to-glucose linkages in the glucomannan. It is doubtful that this disaccharide was a degradation product of a glucan contaminant because such a polysaccharide should have resisted both oxidation with lead tetraacetate and hydrolysis with sulfurous acid. At the very least, a spectrum of cellodextrins would have been expected from the partial hydrolysis of such a polymer.

The absence of a polysaccharide resistant to oxidation was confirmed by the results of the Barry degradation of the oxidized glucomannan.

Finally, the results of boundary electrophoresis supported the assumption that no glucan contaminant was present in the glucomannan. Therefore, it was concluded that contiguous β-1,4 glucose units are present in the glucomannan of white spruce.

The order of these contiguous linkages presented another problem. Statistical calculations by Taylor (54), based on the random distribution of glucose units in a polymer chain of infinite chain length, predicted the following amounts of contiguous β-1,4 glucose linkages in the glucomannan.

Contiguous β-1,4 Glucose Linkages	Per cent of Total Glucose Units	Glucomannan
Cellobiose	22.3	5.1
Cellotriose	7.8	1.8

The results of the present research suggest the existence of only 2.5% of the polymer as cellobiose linkages, and cellotriose linkages appeared to be absent. These percentages are much less than predicted for a purely random distribution of glucose and mannose units in the polymer chains. Conversely, some ordered grouping of sugar linkages is indicated.

RESISTANCE OF MANNOSE UNITS TO OXIDATION

The stable mannose-to-glucose ratio of 1.5 in the oxidized glucomannan suggested that about 45% of the mannose units resisted selective oxidation with lead tetraacetate. This resistance could not have been due to branches on Carbons 2 or 3 because previous studies have shown that little or no branching occurs on these positions (12). Accessibility factors were also discounted

because no unoxidized polysaccharide was found in either the sulfurous acid hydrolyzate or the Barry degradation products of the oxidized glucomannan. In addition, substantial amounts of mannose were found in the hydrolyzate of a sample of ivory nut mannan A which had been oxidized with lead tetraacetate until the rate of oxidation became nil. This finding eliminated the possibility that the glucose units in the glucomannan were responsible for stabilizing the mannose units.

A series of two-stage oxidations was conducted in an attempt to explain the resistance of the mannose units. The first two of these experiments involved treatment of the oxidized glucomannan with fresh oxidant, with and without the intermediate removal of metallic ash. In both cases the amount of additional lead tetraacetate consumed in the second oxidation step was insignificant, and the mannose-to-glucose ratio remained unchanged. Apparently, the inorganic ions in the reaction medium had no effect on the mannose stabilization.

Another two-stage oxidation was performed in which the oxidized polysaccharide was reduced with sodium borohydride between the first and second oxidation steps. This treatment led to the consumption of large amounts of lead tetraacetate in the second oxidation step, and the ratio of mannose to glucose was reduced from 1.5 to 1.2 and 0.7 in separate experiments. Thus, additional mannose units could be oxidized selectively only by destroying the dialdehyde units (or potential dialdehyde units) formed in the first oxidation. Apparently, it was the oxidized units themselves which protected the remaining mannose units from the selective oxidation.

From the considerations discussed above, the following hypothesis was formulated to explain the resistance of some of the mannose units to oxidation.

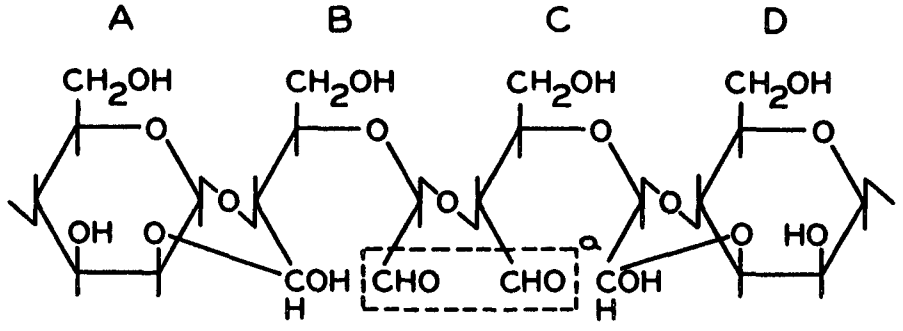
When a glucomannan is oxidized with lead tetraacetate in aqueous acetic acid, the initial attack is selective for the mannose units because of their cis-glycol orientation on Carbons 2 and 3. As the oxidation progresses, however, the oxidized units react with adjacent unoxidized mannose units to render them less susceptible to oxidation. When all of the unoxidized mannose units become stabilized the selective oxidation ceases, and further oxidation is nonspecific.

The qualitative results obtained from oxidized ivory nut mannan A suggests that a like hypothesis should be applicable to that polymer also.

The paucity (or virtual absence) of mannobiose in the sulfurous acid hydrolyzates of oxidized glucomannan and ivory nut mannan A is in accord with the concept of mannose stabilization by an adjacent oxidized unit. If this mechanism were responsible for the stabilization, mannobiose could be isolated only from a glucomannan fragment containing four contiguous mannose units in which the outer units had been oxidized preferentially and had then stabilized the two inner units. This particular sequence of reactions would not occur in the oxidation very often, and the amount of mannobiose surviving should be small.

In addition to the evidence presented above, the infrared spectrum of the glucomannan contained very weak carbonyl bands. Guthrie (55) and others have proposed the formation of hemiacetal rings and hemialdal linkages to account for the lack of carbonyl bands in the infrared spectra of periodate-oxidized carbohydrates. He also cited evidence to show that the six-membered hemiacetal ring was particularly stable in oxycellulose. In addition, Perlin states that the oxidation of sugars with lead tetraacetate in acetic acid proceeds through cyclic hemiacetal formation (24).

Therefore, a possible mechanism for the stabilization of mannose units by adjacent oxidized units could be due to cyclic hemiacetal formation between one of the aldehydes of the oxidized unit and the nearest hydroxyl group of the unoxidized neighbor. The following diagram illustrates such a stabilized structure.



In this structure, a hemiacetal linkage has been formed between Carbon 3 of oxidized Unit B and Carbon 2 of unoxidized Unit A. This linkage destroys the vic-glycol configuration of Unit A, and therefore prevents oxidation. Unit D is also stabilized by a hemiacetal linkage between Carbon 2 of Unit C and Carbon 3 of Unit D. The appended photograph of a Cenco-Petersen[b] molecular model of units A and B (Fig. 6) shows that such a stabilized structure is sterically possible. For purposes of clarity only the essential oxygen (gray) and hydrogen (white) atoms are shown in the photograph.

This photograph indicates that the formation of the hemiacetal is possible by simple rotation of the aldehyde carbon about the glycosidic linkages.

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[a]The free aldehydes shown in the dashed box represent the function only, not the true structure. In fact, these units were probably tied up as acetals or hemiacetals.

[b]This model kit is manufactured by the Central Scientific Company, Chicago.

Very little chain puckering or shortening was required. The same model could also be constructed with the Fisher-Taylor-Herschfelder* atomic model kit. This suggests that van der Walls radii would not inhibit such cyclic hemiacetal formation.

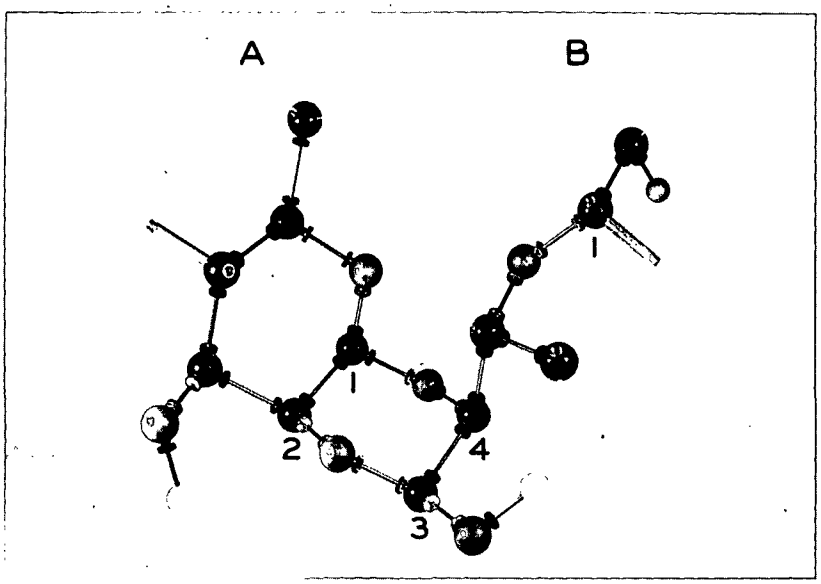

Figure 6. Units A and B of the Oxidized Glucomannan Represented by a Cenco-Petersen Molecular Model

The fact that the cyclic hemiacetal would exist as a stainless, six-membered, stable ring makes more plausible the mechanism proposed for the stabilization of certain mannose units toward rapid selective oxidation. However, the fact that the yields of both mannose and glucose were lower than predicted for selective oxidation, indicates some lability of the stabilizing linkage. The reduced yields, plus the continued slow consumption of oxidant after the

*This model kit is manufactured by the Fisher Scientific Company, New York.

mannose-to-glucose ratio had reached 1.5, suggest that both sugars are oxidized nonspecifically at a much slower rate than in the selective oxidation.

SUGGESTIONS FOR FUTURE WORK

The hypothesis proposed in this thesis states that the resistance of some of the mannose units to oxidation with lead tetraacetate was due to the stabilizing influence of adjacent oxidized units. A study of the oxidation of mannotriose and mannotetraose should provide valuable information on this phenomenon. The finding of resistant mannose units in these oligosaccharides would provide strong support for the hypothesis.

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APPENDIX I

DETERMINATION OF MANNOSE-TO-GLUCOSE RATIO BY REFLECTANCE METHOD

Several workers have shown that it is possible to determine the concentration of sugars in a hydrolyzate by direct measurement of the reflectance of the colored areas on a chromatogram (30, 56-58). The most useful relationship is a plot of sugar concentration versus the Kubelka-Munk scattering coefficient (K/S) which is linear over an extended range of concentrations. Since the relationship of K/S to sugar concentration is linear, the relationship of the ratio of the scattering coefficients to the ratio of the two sugars in solution should also be linear.

The method developed was based on the work of Jeffery and co-workers (30).

Aliquots (15 µl.) of sugar solutions containing known mannose-to-glucose ratios were spotted 1-1/2 inch apart on S & S no. 598 chromatography paper. Total sugar concentration per spot was 150 µg. All paper used for quantitative estimations had been dipped in distilled water and dried without tension in order to relieve strains in the sheet.

The chromatograms were irrigated for 16 hours with a solvent composed of butyl acetate, pyridine, 95% ethanol, and water (8:2:2:1). After irrigation the sheets were dried in a forced air hood for at least one hour.

The dried chromatograms were dipped in the color developing solution composed of 0.5% aniline and 1.5% monochloroacetic acid dissolved in diethyl ether. The sheets were then hung for three hours in a humidity cabinet. Humidification was found to improve the chromatograms in two ways:

1. Background color of humidified chromatograms was considerably lighter than nonhumidified ones.

2. Sugar spot densities were intensified markedly by the humidification step.

Reflectance measurements were made with a Welsh Densichron using a 0.25 inch pickup boot with a yellow filter. The background reading between spots was averaged and set at 100% reflectance so that net reflectance was measured. Minimum reflectance of a given spot was obtained. Reflectance was converted to Kubelka-Munk scattering coefficient $\underline{K}/\underline{S}$ with suitable tables (59). Results were plotted as

$$\frac{\underline{K}/\underline{S}\ (\text{Mannose})}{\underline{K}/\underline{S}\ (\text{Glucose})} \text{ vs. } \frac{\text{Weight Mannose}}{\text{Weight Glucose}}$$

The data from 15 independent chromatograms are listed in Table X. The major conclusions drawn from these experiments are:

1. Plots of $(\underline{K}/\underline{S}_m)/(\underline{K}/\underline{S}_g)$ vs. wt. mannose/wt. glucose are linear over the mannose-to-glucose weight ratio range of 0.33:1 to 3.0:1.

2. The plots are independent of total sugar concentration within this range if the concentration of the minor sugar component is greater than 20 μg. and the concentration of the major sugar component is less than 120 μg.

3. The ratio plots contain less variance about the regression line than individual plots of $\underline{K}/\underline{S}$ vs. sugar concentration.

4. The linear relationship is dependent on unknown paper properties. The slopes of the regression lines vary from sheet to sheet.

In the actual determinations, hydrolyzates were spotted along with known solutions of mannose and glucose in different ratios on duplicate chromatograms. Reflectances were measured, and the ratio of the scattering coefficients

were calculated for each sample. Graphs were constructed for each chromatogram using the known samples. The mannose-to-glucose ratios of the unknowns were then determined from these plots. Duplicate determinations were averaged.

TABLE X

VALUES OF THE RATIOS OF THE SCATTERING COEFFICIENT OF MANNOSE ($\underline{K}/\underline{S}_m$) TO THAT OF GLUCOSE ($\underline{K}/\underline{S}_g$) FOR STANDARD SOLUTIONS OF MANNOSE AND GLUCOSE OBTAINED FROM REFLECTANCE MEASUREMENTS WITH THE DENSICHRON

Mannose-to-Glucose Ratio $\frac{\underline{K}/\underline{S}_m}{\underline{K}/\underline{S}_g}$ in Solution				Standard Error About Regression Line	Standard Deviation About Regression Line
0.33	1.0	2.0	3.0		
0.25	0.73	1.65	2.46	0.000	0.00
0.22	0.78	1.65	2.37	0.015	0.12
0.24	0.82	1.67	2.51	0.010	0.10
0.40	0.85	1.39	1.84	0.005	0.07
0.31	0.85	1.51	2.14	0.000	0.00
	0.79	1.50	2.34	0.010	0.10
	0.79	1.56	2.39	0.000	0.00
	0.83	1.61	2.08	0.000	0.00
	0.83	1.53	2.26	0.010	0.10
	0.78	1.51	2.42	0.000	0.00
	0.86	1.37	2.29	0.030	0.17
	0.79	1.69	2.68	0.000	0.00
	0.82	1.76	2.46	0.000	0.00
	0.73	1.49	2.94	0.070	0.26
	0.86	2.10	3.10	0.030	0.17
	Expected Standard Error			0.010	
	Expected Standard Deviation				0.10

APPENDIX II

LEAD TETRAACETATE CONSUMPTION CURVES

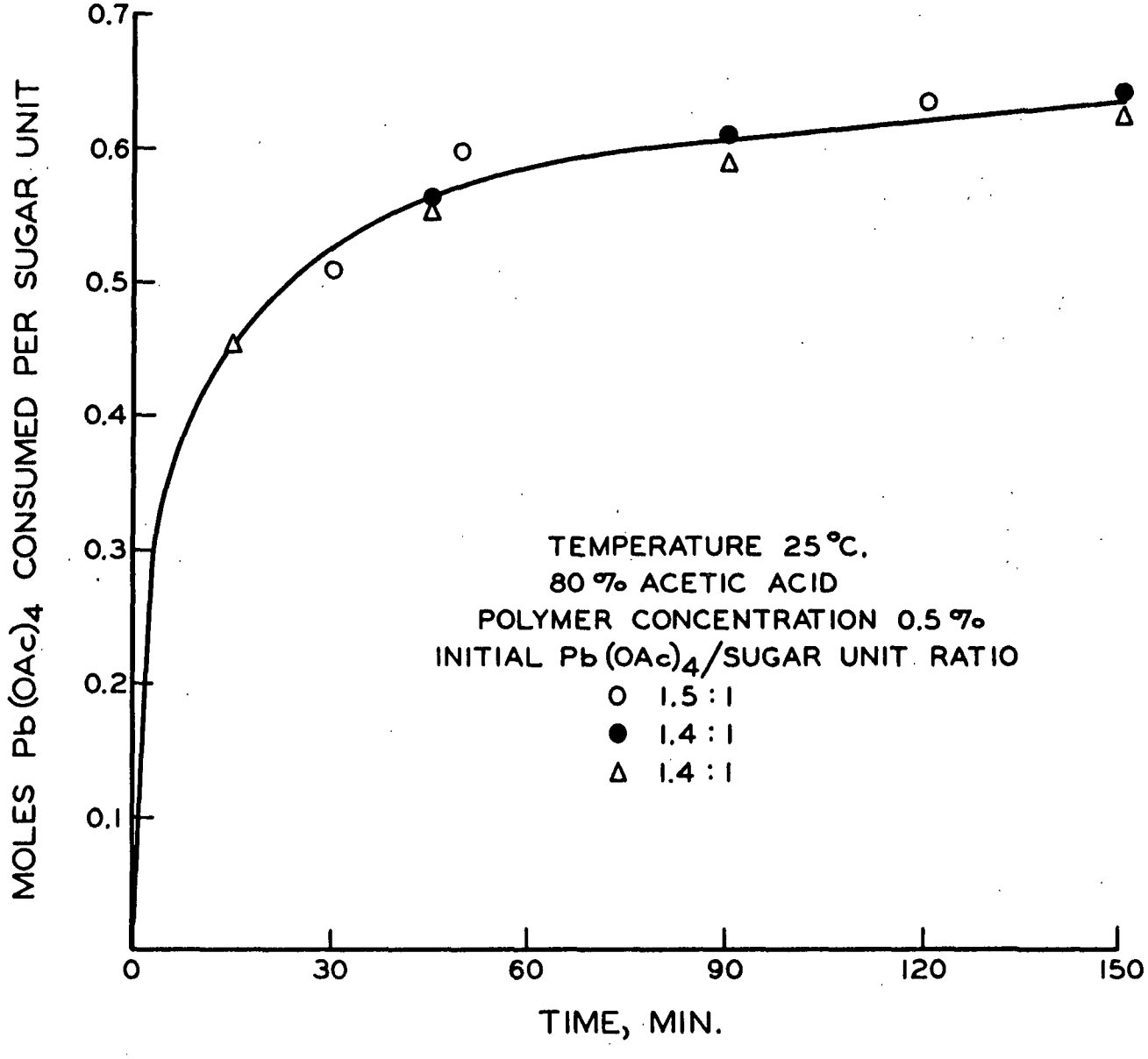

Figure 7. Large-Scale Oxidations of Glucomannan

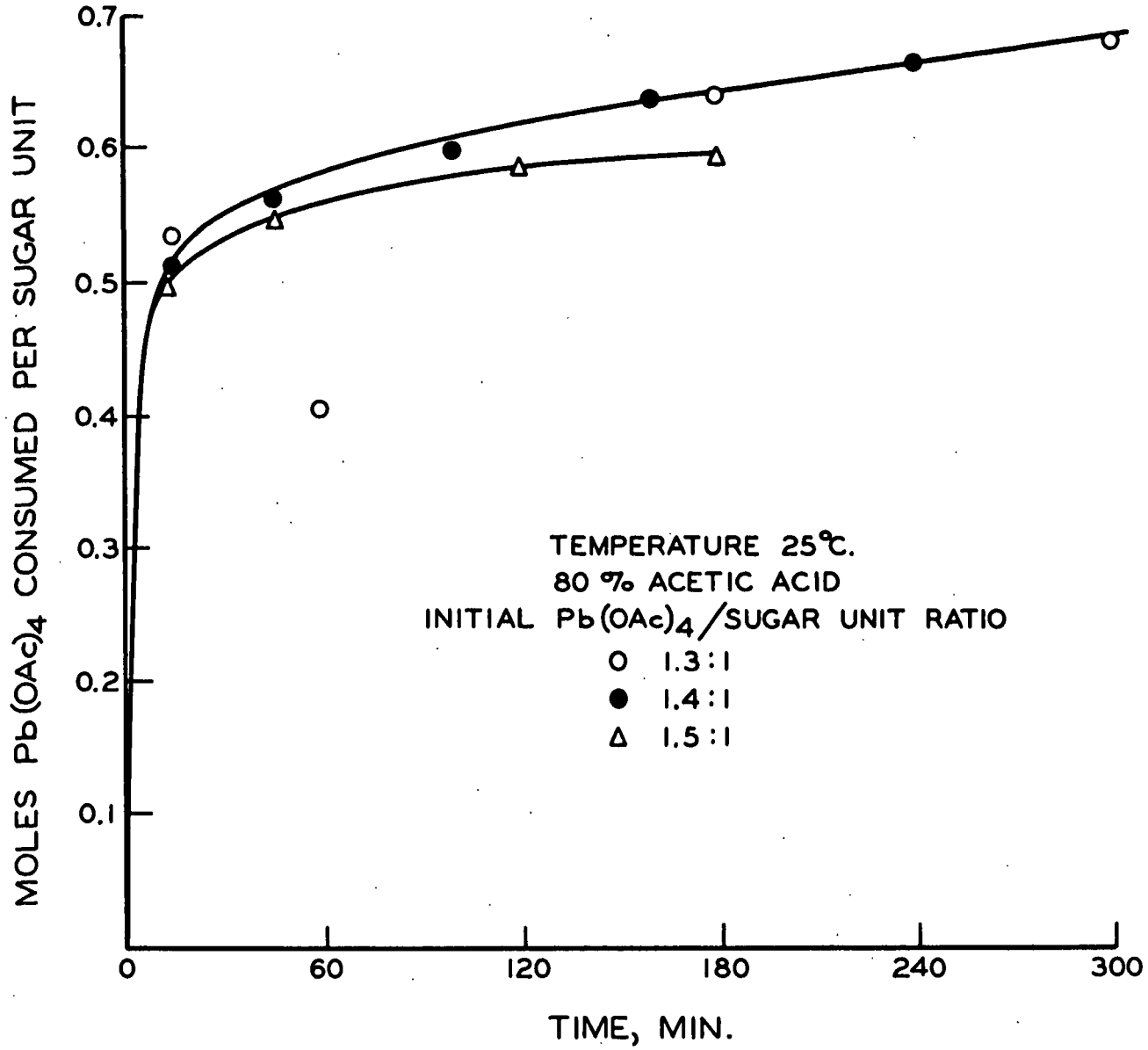

Figure 8. Reproducibility of Oxidation at 0.5% Polymer Concentration

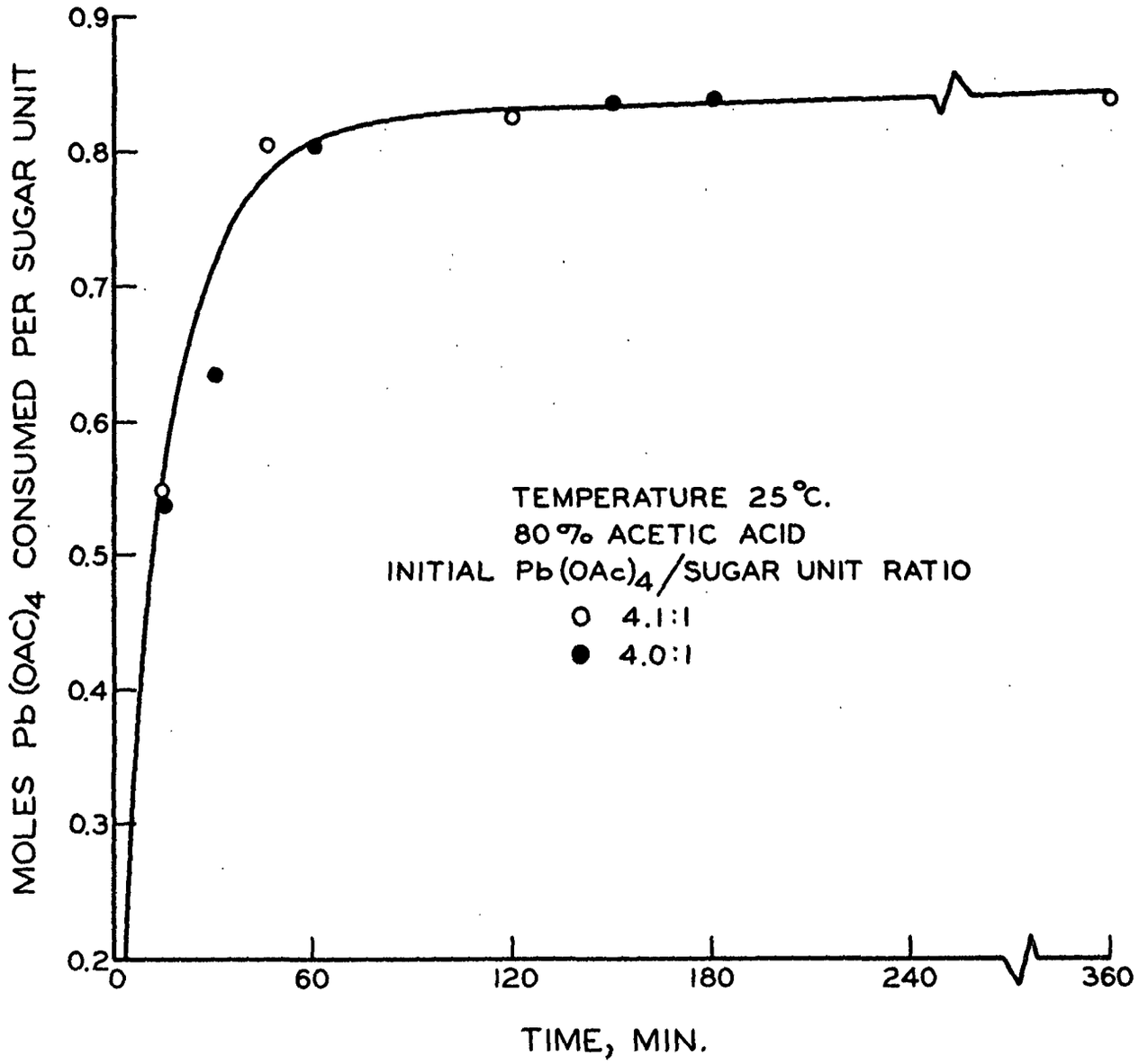

Figure 9. Reproducibility of Oxidation at 0.2% Polymer Concentration