THE GLUCOMANNANS OF SITKA AND BLACK SPRUCES

BY

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ABSTRACT

A study was made of two glucomannans, one isolated by alkaline borate extraction of Sitka spruce wood and the second from black spruce. These were methylated by the Hakomori procedure employing sodium hydride in dimethyl sulfoxide. Considerable experimentation was done to determine the best conditions for methylation and to demonstrate the utility and practicability of analysis by these methods. Some inferences regarding the structure of the glucomannans are drawn from the methylation data.

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INTRODUCTION

A large variety of polymeric carbohydrate substances are isolable from the woody portions of angiosperm and gymnosperm species. The term hemicellulose has been traditionally applied to such substances whether homo- or heteropolymeric in composition but excluding cellulose and starch. The literature appertaining to the general properties and structures of hemicelluloses is very extensive and well documented (1-3). Therefore only a very brief disucssion will be given here. Three principal classes of polysaccharides are found to make up the hemicellulose content of the softwood trees (conifers or gymnosperms). These are: glucomannan, galactoglucomannan and arabinoglucuronosylxylans.* In the spruces which are to be considered here the three types are found in the approximate proportion of 15%, 5% and 16%, respectively, based on the original weight of dried wood. A fourth class may be included, that of the water soluble or water

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* These acronyms for the polysaccharides indicate the principal sugars to be found in each class. Other sugars may be found in small amounts, for example, glucomannans of softwoods contain a very low percentage of galactose and some arabinoglucuronosylxylans contain 4-O-methyl-D-glucuronic acid. For convenience the abbreviations GM, GGM and xylan have been commonly used to refer to the three types in this thesis.

extractable polysaccharides. They are recovered by direct water extraction of the milled wood (before delignification) in a yield of from 1 to 2% and consist mainly of arabinogalactans (4).

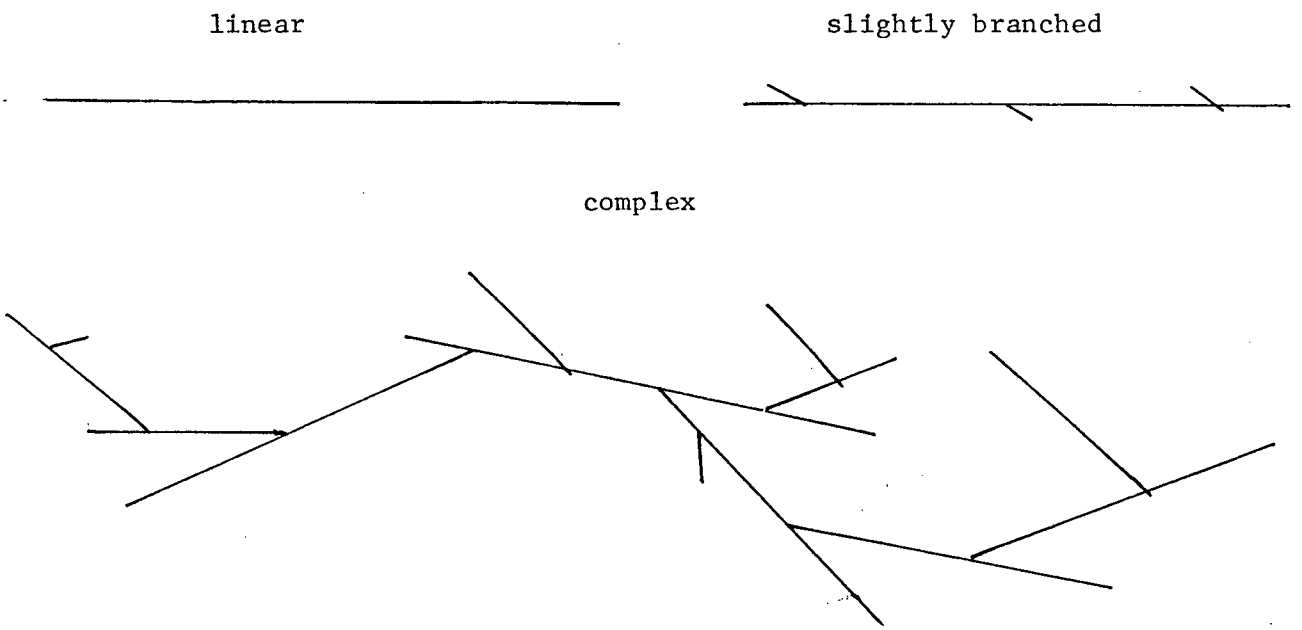
In trees of the hardwood (angiosperm) group the galactoglucomannan class is generally absent and a roughly corresponding increase in the amount of xylose polymers is observed. It is interesting to note that in the species Sequoia sempervirens - a tree which is considered an "evolutionary intermediate" between true angiosperm and gymnosperm types - only a very small amount of galactoglucomannan is found (5).

Any carbohydrate polymer, regardless of whether it consists of a unique anhydro sugar unit or several may be either branched or linear. A branched polymer may have either simple one unit side chains extending from a linear backbone or a multiply branched system in which no main chain can be defined. This is illustrated in Fig. 1. Highly branched systems are quite uncommon in wood hemicelluloses but are more frequently encountered in gum exudates.

With this in mind it is clear that any analysis of a hemicellulose must do more than characterize the type of sugar residues present. The sequence of the different monosaccharide units, the number and type of end groups and the character of the branch points must be demonstrated.

The most general method of obtaining such information is by methylation of all free hydroxyl functions in the polymer. Subsequent hydrolysis liberates a series of partially methylated sugars, that have unblocked hydroxyl groups corresponding to the positions of

Figure 1. General Types of Polysaccharides



linkage in the original polysaccaride. This method is a development of the procedures originally used to solve the structures of the cyclic glycosides.

The earliest practical procedure for methylation of polysaccharides was that developed by Haworth (6). Since then many refinements have been made in both techniques of methylation and methods of analysing the resultant methyl sugars. A considerable improvement in the detail of analysis is therefore now possible. The reasons underlying this and the methods that may be employed will be described more fully in the discussion.

With this in mind it was decided to undertake an analysis of a series of softwood hemicelluloses. Since the woods with which we are working are commercially important species considerable work has already been done on their hemicelluloses or at least those of closely related species (7-11,60). Thus in many cases one has a considerable amount of information available about the gross structure of the polysaccharides to be obtained. Our work has therefore been directed toward extending the knowledge of end groups and branch points present in the polymer. As mentioned in the Abstract, this thesis describes the investigation of analytical and preparative methods, and the results obtained upon the glucomannans from Sitka spruce (Picea sitchensis) and black spruce (Picea mariana).

DISCUSSION

1. General.- The traditional method of analysis of wood polysaccharides consists first of isolation and purification followed by determination of sugar ratios, methylation, analysis of the resultant methylated sugars and, optionally, periodate degradation.

In this study periodate cleavage was not employed as the technique is better designed for confirmation of methylation results in gross structure determinations. Periodate degradation involves cleavage of any vicinal (unblocked) hydroxyl functions in the polymer chain. Analysis of the resultant fragments enables one to reconstruct the types of linkage and branch points originally present. The inherant accuracy is limited by over oxidation (i.e. the amount of periodate consumed becomes greater than that required for stoichiometry with the number of cleavable sites), thus restricting the degree to which the original structure can be inferred (12,13).

2. Isolation.- The isolation and purification of wood polysaccharides is a problem which appears to have no perfectly satisfactory solution. Two basic difficulties may be defined. The hemicelluloses are very firmly held in a rigid cell structure in the wood itself (15,59). They are, if not chemically, at least very strongly

hydrogen bonded to the lignin in the wood (16). Thus very strong reagents are required to solubilize them. This raises the possibility of degradative elimination (18) or alkaline peeling reactions (17) occurring and certainly any base sensitive substituents such as acetyl (which some wood polysaccharides are known to contain) (19-21) are quickly removed. Secondly, the problem of separating each hemicellulose from the others and establishing its purity is not trivial (14).

Chemical methods are generally of little use here, as with the exception of the separation of acidic polysaccharides by reaction of the carboxyl group, chemical differentiation between hemicelluloses is not sufficiently great. Physical methods are therefore generally employed; principally preferential extraction and precipitation. A number of fractionation schemes have been proposed to accomplish the required separation (11,22,23). One of the most useful and generally applicable is that of Timell (24). This method was used to isolate the glucomannans of black and Sitka spruces and is described in detail in the experimental section.

The general scheme for the isolation of the polysaccharides may be seen in Fig. 2 and the more detailed procedure for purification of the crude glucomannans is shown in Fig. 3. The method of separation of galactoglucomannan and xylan in the 24% potassium hydroxide extraction mixture will not be discussed further and is shown only for clarity.

Upon completing such an isolation procedure it would be desirable if some means could be employed to rigorously demonstrate the

Figure 2. Isolation of Glucomannans.

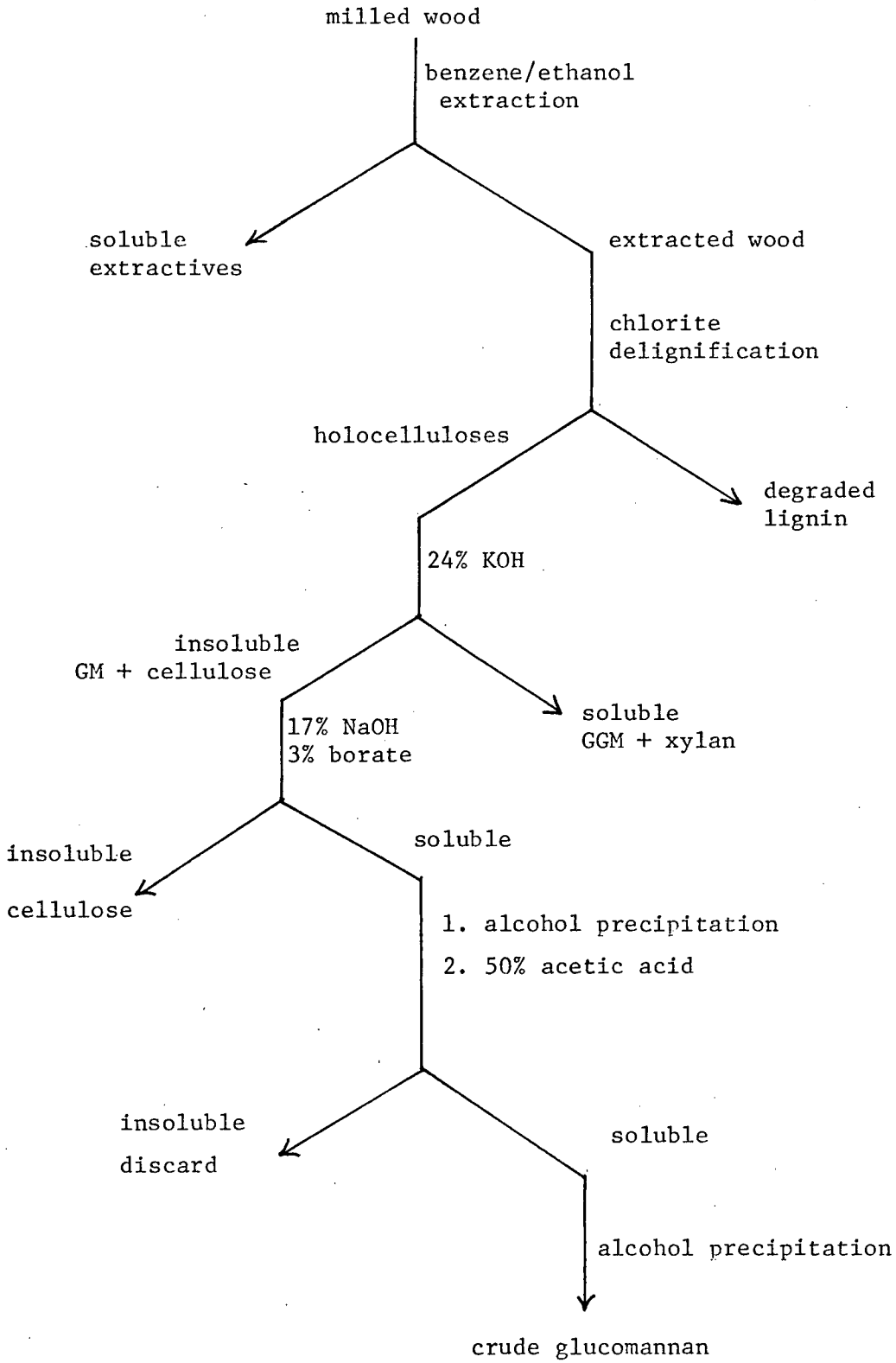
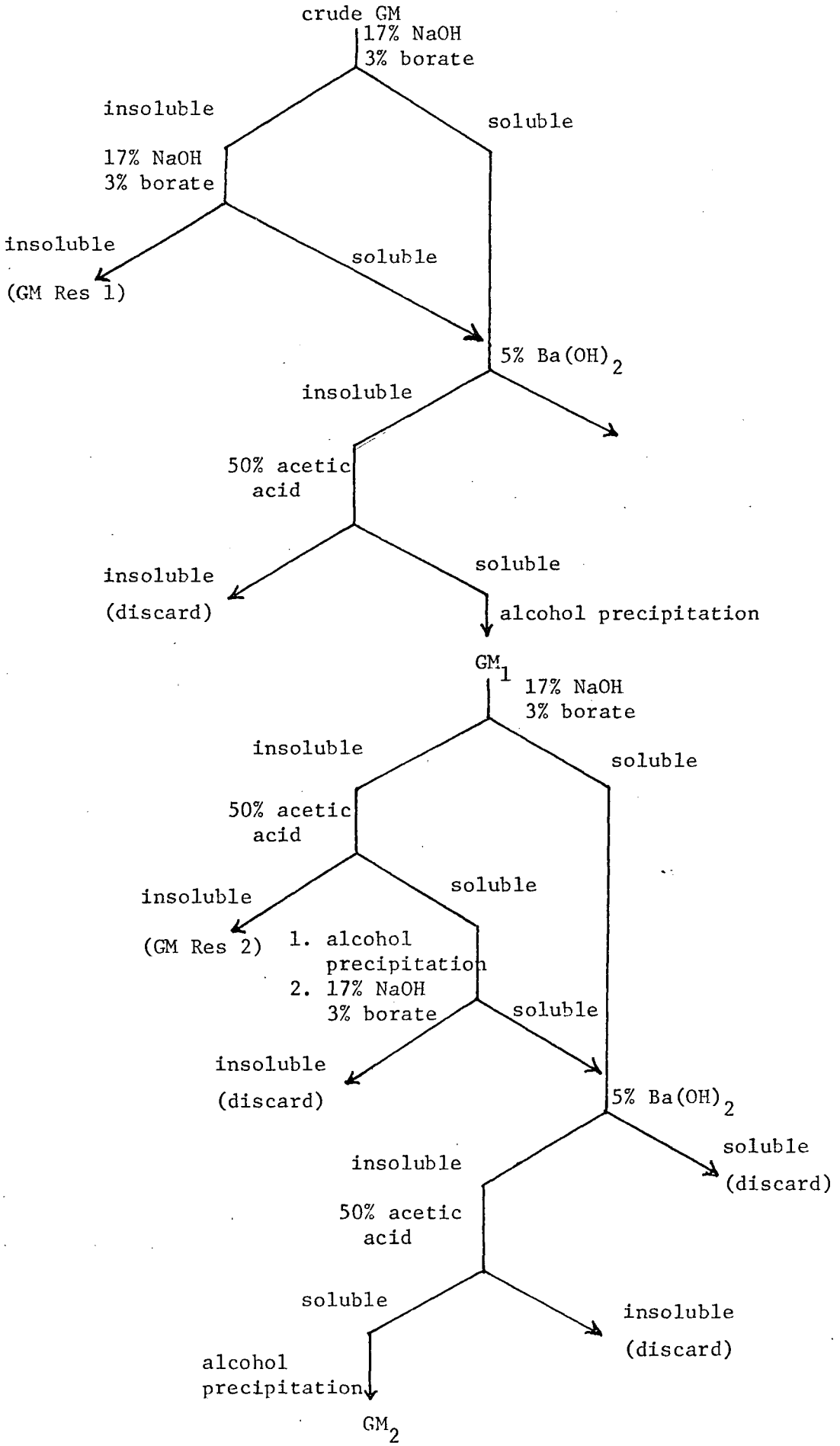


Figure 3. Purification of Glucomannans.



purity and homogeneity of the product. This, as mentioned before, is not easily accomplished for polymeric carbohydrates and we have been forced to rely on a variety of methods, none of which are entirely satisfactory. These are as follows: gel filtration chromatography, sugar analysis, optical rotation, and moving boundary electrophoresis. The first of these was attempted in our laboratory on polysaccharides thought to be homogeneous and on deliberate mixtures of hemicelluloses (GGM's and xylans) on Sephadex from G—25 to G—200 (25). Little or no useful separation was achieved. The ratios of constituent sugars and the optical rotation of the polymer both as free and methylated polysaccharide provide useful clues (1). If relative amounts of constituent sugars remain the same between successive purifications and in substantial agreement with those obtained for various fractions of the methylated polymer a strong indication of homogeneity is provided. Similarly constant optical rotation will indicate essential homogeneity. The results in the experimential section show that for the glucomannans at least, quite a high degree of purity is achieved. It is probable that in all cases, however, complete homogeneity is not achieved as such highly hydroxylated polymers are strongly co-precipitated and/or co-solubilized (26). An example of this is the observation that the residue obtained when dissolving the crude glucomannan in borate/alkali consists largely of cellulose, which would not have been expected to dissolve under the conditions of extraction.

The technique of moving boundary electrophoresis (Tiselius) has been used successfully several times to establish the homogeneity of

polysaccharides (27-29). It was attempted with one of our polysaccharides and the results were encouraging but not conclusive. Unfortunately, early in this investigation the only available Tiselius apparatus failed and was not replaced. It is likely, however, that by using preparative Tiselius techniques small quantities of rigorously purified polysaccharides could be isolated. These would be sufficient for methylation by the Hakomori procedure and to provide a sample of high quality for gas-liquid chromatographic analysis once the retention times of the sugars to be expected had been established.

Sugar ratio determinations on the unmethylated polysaccharides were done first by paper chromatography to indicate the principal sugars present and then by two gas-liquid chromatographic methods. For gas chromatography the free sugars were converted to their trimethylsilyl ethers in pyridine (30,31) or reduced to the alditols with sodium borohydride and acetylated (32). Although both methods give results in quite close agreement some difficulty is encountered in determining small percentages of galactose. When silyl ethers of the sugars are prepared mixtures of anomers are formed. The total amount of any sugar in a mixture is calculated from the peak size of some anomer which is resolved completely from peaks contributed by any other sugar. This implies that silylation must be done under such standard conditions that the anomeric ratios remain constant (31). In the case of galactose the peak measured constitutes only about 28% of the total galactose and therefore a small error in counting or equilibrium shift may become quite significant.

In chromatography of the alditol acetates it is difficult to

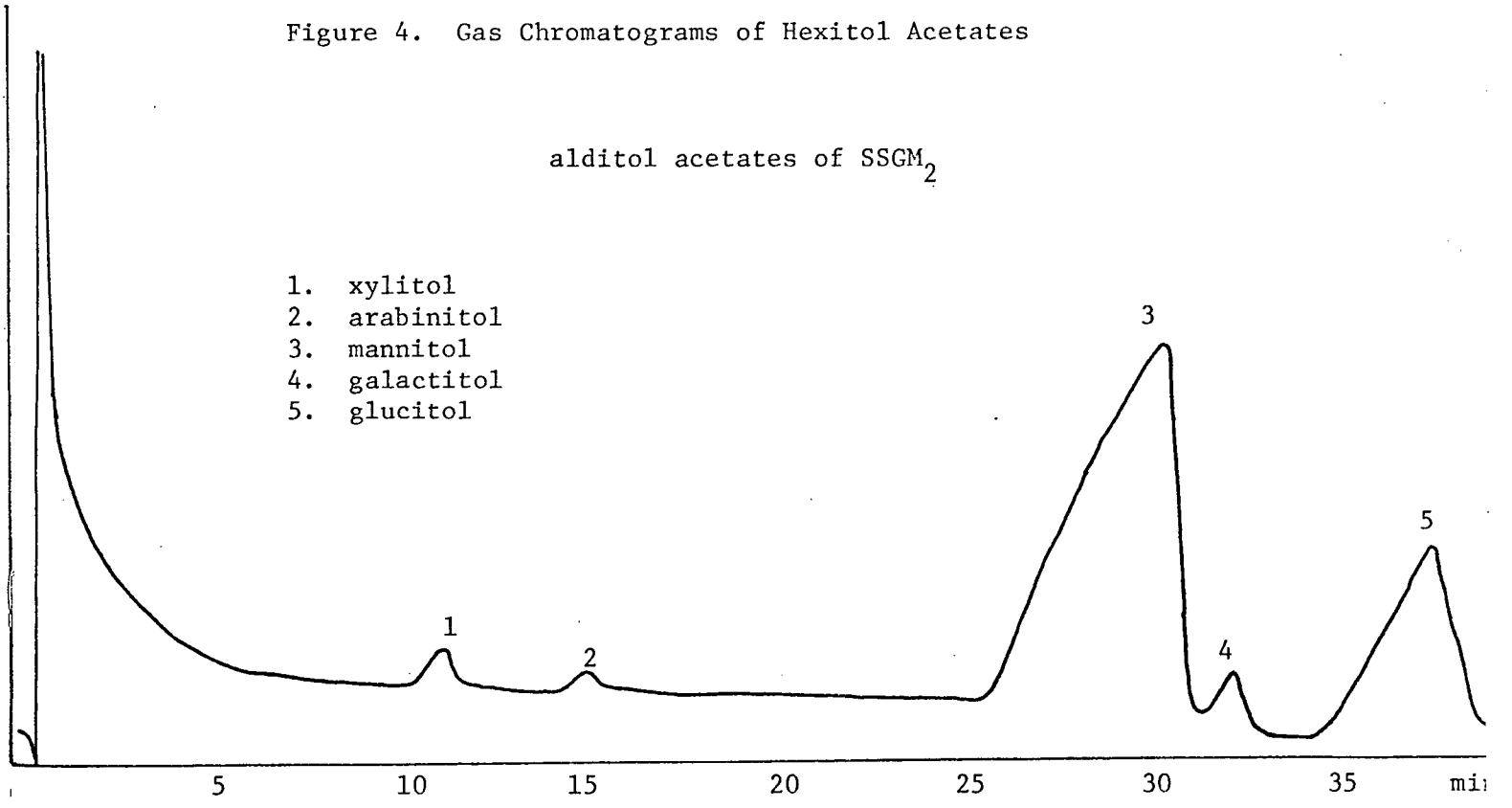
completely resolve galactitol from mannitol in the presence of large amounts of the latter. A series of experiments with standard mixtures showed that in spite of the difficulties of resolution reasonable accuracy and reproducibility can be obtained. The amount of galactitol present may, if necessary, be estimated if it is very small in proportion to the amount of mannitol present. Under these circumstances it may happen that the galactitol appears only as a shoulder on the mannitol peak. Two sample chromatograms are reproduced in Fig. 4.

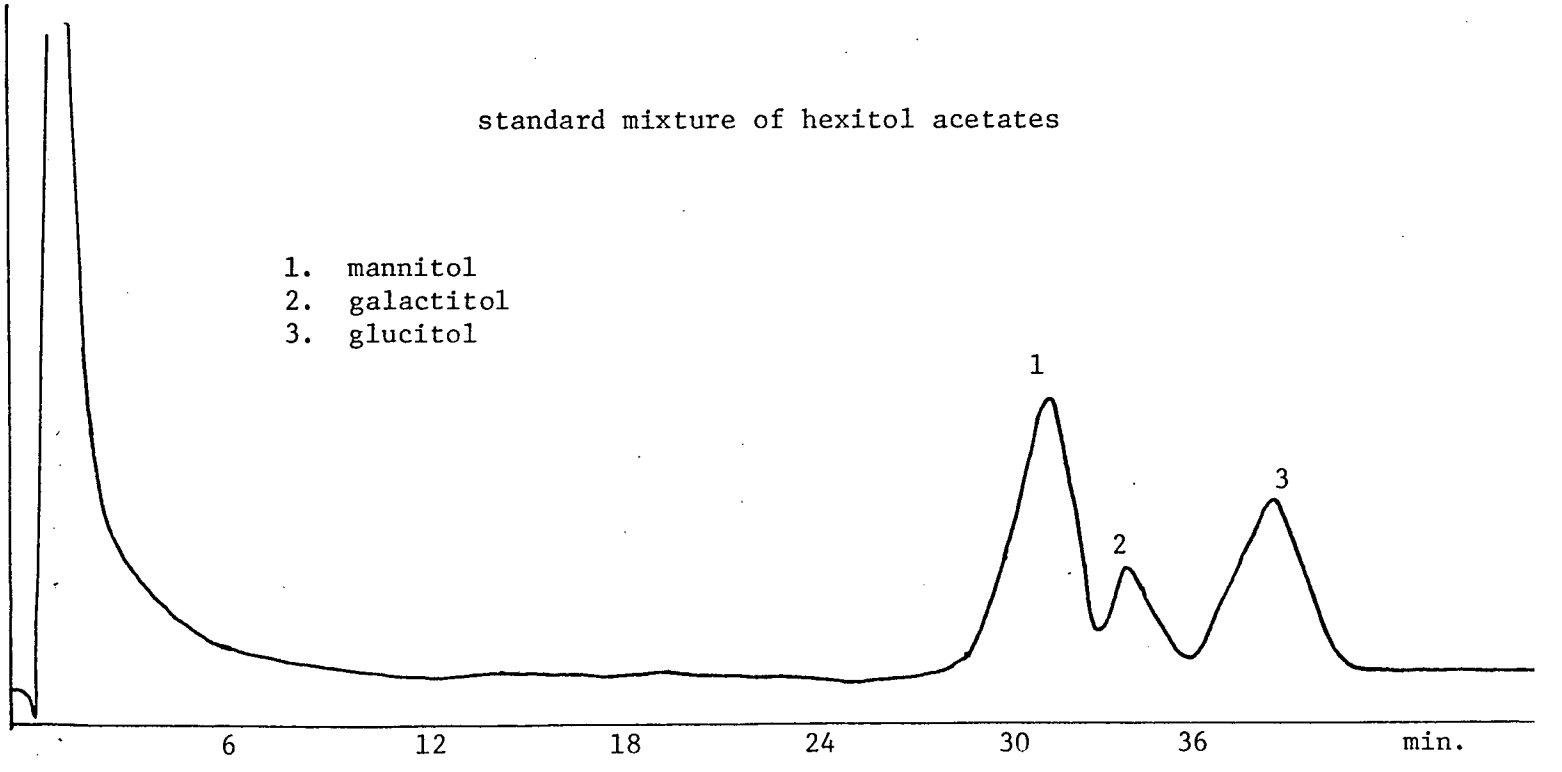
Considerable experimentation was done to determine the optimum conditions for gas liquid chromatography of both alditol acetates and partly methylated alditol acetates. All possible parameters of solid support, percentage of liquid phase, flow rate, initial temperature and program rate were varied.* A four foot by three sixteenths inch column was used for alditol acetates and an eight foot by three sixteenths inch one for partly methylated alditol acetates.

3. Methylation.- The methyl ether functionality is one of the few groups which can be put on the hydroxyl functions of a polysaccharide and still survive the rather drastic conditions required for subsequent hydrolysis. For this reason it has been

* Considerable improvement was obtained when using non-acid washed Chromosorb W in place of Gas Chrom Q as solid support with a 3% coating of ECNSS-M as liquid phase. This modification was suggested by Shaw's work (33) in which he compared results obtained for the g.l.c. of carbohydrate derivatives on a wide range of liquid phase and solid support systems.

Figure 4. Gas Chromatograms of Hexitol Acetates





consistently employed for over seventy years and still forms the basis for most polysaccharide analysis. Numerous methods have been developed in an attempt to improve yield, completeness, and ease of methylation. Four methods have been commonly employed with considerable success. Haworth's (6) and Purdie's (34) methylations may be considered complementary. The former employing aqueous sodium hydroxide and dimethyl sulfate may be used to partly methylate an unsubstituted polysaccharide insoluble in organic solvents followed by the latter using silver oxide in refluxing methyl iodide to complete the substitution. More recently the Kuhn procedure (35,61) has been employed using barium hydroxide/barium oxide in dimethyl formamide with methyl iodide. The latest and most important development in methylation is the Hakomori technique using dimethyl sulfoxide, sodium hydride and methyl iodide (36,37). It has to a great extent revolutionized the procedure of methylation as material may be brought to a very high degree of substitution in one treatment involving only a few steps. The yields obtained are extremely high, often close to theoretical. Thus, much smaller quantities of material can be used. The reaction is carried out by dissolving (or suspending if it is not soluble) the polysaccharide in dimethyl sulfoxide. A solution of sodium hydride in dimethyl sulfoxide is prepared:

$$NaH + CH_3\overset{\overset{O}{\|}}{-S}-CH_3 \longrightarrow Na^+ + \underset{I}{CH_2\overset{\overset{O}{\|}}{-S}-CH_3} + H_2\uparrow$$

The so-called dimsyl ion (I) is then added and allowed to react to form the alkoxide of the carbohydrate hydroxyl functions. Methyl

iodide is then added slowly and with cooling. Although the reaction is straight forward several points have been observed which significantly improved our results.

1. The polysaccharide should be in such a form as to permit maximum solution or at least maximum solvent penetration. Insoluble polysaccharides will require at least two methylations.
2. Polysaccharide concentration should not exceed 5% as otherwise gel formation may be so intense as to prevent proper mixing.
3. Air (oxygen) and moisture should be excluded to a reasonable degree.
4. The reaction of dimsyl ion with polysaccharide hydroxyl functions is not instantaneous and improved results are obtained if the base is allowed to react 4-6 hrs with the polysaccharide (the mixture should be stirred if it is inhomogeneous) before the methyl iodide is added.
5. It was found on analysis of sodium hydride oil dispersion mixtures that had been opened several times that the actual amount of dimsyl ion formed on reaction with dimethyl sulfoxide was only 45% of the expected value. Thus a check on the amount of reagent necessary to generate a two-fold excess of dimsyl ion is advisable.

Workup of the methylated polysaccharide is extremely easy. The dimethyl sulfoxide solution is poured into a large excess of water and dialysed until all dimethyl sulfoxide is gone. The solution

or suspension may then be either extracted with chloroform, freeze dried or evaporated to dryness under vacuum.

Although very high degrees of methyl substitution are obtained by one Hakomori methylation we were unable to obtain complete substitution. In order to avoid repeated exposure to the rather drastic conditions of the Hakomori methylation (38) it was decided to complete the substitution with Purdie's Reagents (methyl iodide, silver oxide). This approach was suggested by Wallenfels (35) in his work in which he reports that a combination of methylation techniques is more effective than repeated application of one method.

Fractionation with petroleum ether/chloroform mixtures was carried out on the crude methylated polysaccharide. Having excluded degraded and undermethylated material by this means the larger part of the sample was given a Purdie methylation. The recovered material was again fractionated and analyzed for methoxyl. The specific details of procedures followed for each polysaccharide may be seen in the experimental section. It may be noted however, that the methylated polysaccharides extracted by a 20% chloroform/petroleum ether mixture contained a detectable amount of 2,3-dimethyl xylitol whereas the 15% fraction contains none. In a similar analysis of ponderosa pine glucomannan which contained xylose detected by paper chromatography before methylation the quantity of dimethyl xylitol in the 20% fraction was quite significant but almost nil in the 15% fraction. Thus it may be seen that a certain separation of polysaccharide types does occur during this extraction.

Methylated polysaccharides were examined by IR spectroscopy in

carbon tetrachloride solution to determine the degree of substitution (Figs. 4-6). When a polymer with little or no absorption at 3500 cm^{-1} was obtained duplicate methoxyl analyses were done by the Zeisel method.

4. Hydrolysis.- Several methods have been used extensively for the hydrolysis of methylated polysaccharides (39,40). It is important to achieve complete hydrolysis with as little demethylation or degradation as possible. We have routinely used two methods both of which give acceptable results. The polysaccharide is first solubilized in either 90% formic acid or 72% sulfuric acid at 0°C, then refluxed in 1 N sulfuric acid for six hours. To circumvent possible losses of more volatile methyl sugars the neutralized hydrolysate was extracted with chloroform prior to concentration. The chloroform extract can be added to the sugars from the aqueous fraction and the whole evaporated at a low temperature.

5. Analysis.- When the mixture of methylated sugars derived from the original polysaccharide is obtained some method of analysis must be selected. One of our objects in this work was to apply Lindberg's method of reduction of the sugars to their alditols, gas chromatography of the alditol acetates and identification by mass spectrometry to polysaccharides containing three different hexoses (38,41-43). Once one reduces the sugars to their alditols there is only one gas-liquid chromatograph peak obtained for each sugar. This is a distinct advantage over procedures employing methyl glycosides (44,47), silyl derivatives (30,31) or the free sugars, wherein mixtures of anomers are obtained. In addition, the mass spectrometric

results make it possible to determine the pattern of methoxyl substitution on very small amounts of sample where the preparation of a crystalline derivative would be impractical. From the information of gas-liquid chromatographic retention time and fragmentation patterns, the identity of the alditol can usually be unambiguously confirmed. In addition the partly methylated alditol acetate can easily be demethylated by the method of Bonner and Bourne (48). The free alditol can then be reacetylated and identified by its gas-liquid chromatography retention time as fully acetylated hexitol. The alditol acetates of galactose, glucose and mannose crystallize quite readily and a melting point may be taken if sufficient sample can be collected from the g.l.c. The same procedure can be employed to greatly reduce the possibility that any gas-liquid chromatograph peak may be a combination of two different partly methylated sugar alditols having virtually identical retention times. For example, 2,3,4-tri-O-methyl-D-mannitol, Rt = 2.48 (38) would not be resolvable from 2,3,4-tri-O-methyl-D-glucitol, Rt = 2.49 and could not be distinguished by mass spectrometry. Gas-liquid chromatography as the unmethylated alditol acetates would, however, indicate the presence of both glucitol and mannitol.

Because of our particular interest in the detection of end groups we have also done gas-liquid chromatography on the methyl glycosides of the tetramethyl sugars obtained on hydrolysis. This method permits a positive demonstration of the presence of 2,3,4,6-tetra-O-methyl-D-glucose in the presence of 2,3,4,6-tetra-O-methyl-D-mannose whereas these two are extremely difficult to resolve as alditols and the very

small amounts present make the collection of sufficient sample for demethylation quite tedious.

Although the mass spectrum gives a unique fragmentation pattern for each different partially methylated hexitol, one complication may arise. Because the mass spectrometer does not distinguish stereochemical differences, there is loss of information upon reduction. For example, a 2,3-dimethyl and 3,4-dimethyl pentose or a 3,5- and 2,4-dimethyl hexose would give the same mass spectrum after reduction. In practice confusions of this type rarely arise and can be resolved by comparison of gas-liquid chromatography retention times or reduction with borodeuteride which specifically labels the original C1 end of the sugar (43).

EXPERIMENTAL

1. General.- To obtain polysaccharides in an easily manipulated form of highest solubility the technique of solvent exchange was routinely employed. A solution of the polysaccharide was poured with constant stirring into four volumes of alcohol containing 5-7% glacial acetic acid. The precipitate so formed was washed once with alcohol/acetic acid, twice with alcohol, twice with acetone and twice with diethyl ether. The remaining solvent after centrifugation and decantation was removed by heating in a stream of warm air accompanied by constant trituration. All alcohol used for these and similar precipitations was commercial 1K formulated from 95% ethanol/5% methanol.

Paper chromatography was done at room temperature on Whatman #1 paper in 8:2:2 ethyl acetate/pyridine/water for twenty-four hours, 4:1:3 butanol/ethanol/water for twenty-four hours or methyl ethyl ketone/water azeotrope for five hours (49,50). The results are recorded in Table I.

Determination of the methoxyl content of methylated polysaccharides was done in duplicate by the Zeisel method and appropriate allowance made for any ash found. The ash was assumed to be inert material as no uronic acids could be detected in the hydrolysates of the polysaccharides and it cannot therefore have arisen from cations

present in combination with these. As a preliminary to any methoxyl determination and as a general monitoring of the progress of methylation IR spectra were routinely run on the methylated polysaccharides in carbon tetrachloride solution at a concentration of 7-10%. Sample spectra for SSGM's may be seen in Figs. 5-7. They show that if care is taken to exclude traces of moisture an excellent idea may be obtained of the degree to which substitution has proceeded. Samples were dried under vacuum at 60°C for eight hours prior to analysis.

Gas-liquid chromatography was done on an F & M 720 equipped with a thermal conductivity detector. Integration of peak area was done using an Infotronics CRS-100 integrator. Column packings and program parameters are listed in Table II. Column packings were prepared by dissolving the liquid phase in chloroform or acetone, mixing thoroughly with the solid support and then evaporating the solvent on a rotary evaporator. The columns were manually packed with vibration and tamping and conditioned at 100°C and a low flow rate for twenty-four hours before use. All rotary evaporations were performed at 35-40°C in vacuo except where otherwise noted.

Deionizations were done by passing the solutions through a 12 cm column of IR 120 and eluting with 4 to 5 volumes of distilled water.

2. Preliminary treatment of Sitka spruce.- Sitka spruce sawdust (500 g) was extracted in a Soxhlet extractor in two lots of 250 g with 2000 ml of a 2:1 benzene/ethanol mixture until the extract was colourless. The extracted sawdust was then shaken for

Figure 5. IR Spectrum of $SSGM^{302-368}$

methoxyl = 39.1%

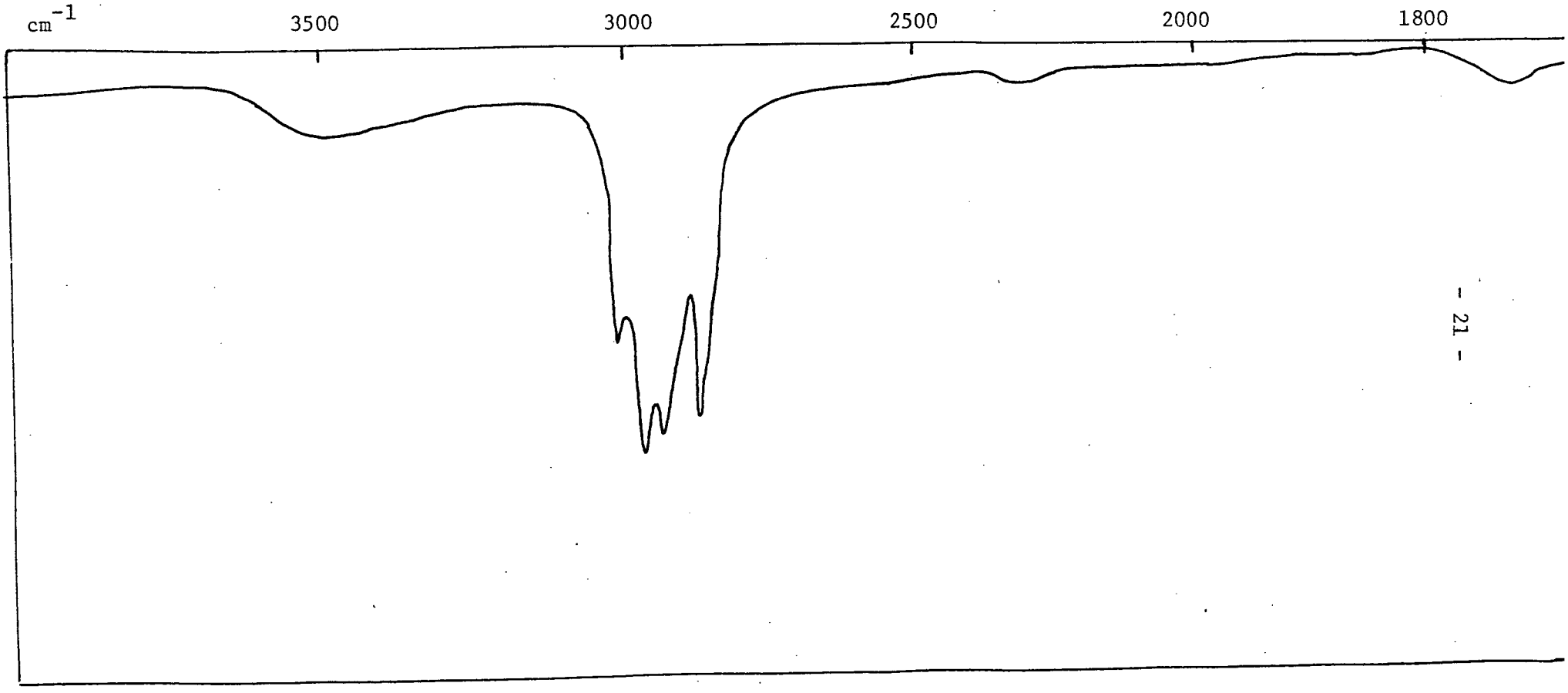


Figure 6. IR Spectrum of $SSGM_{15}^{302-308}$

methoxyl = 44.4%

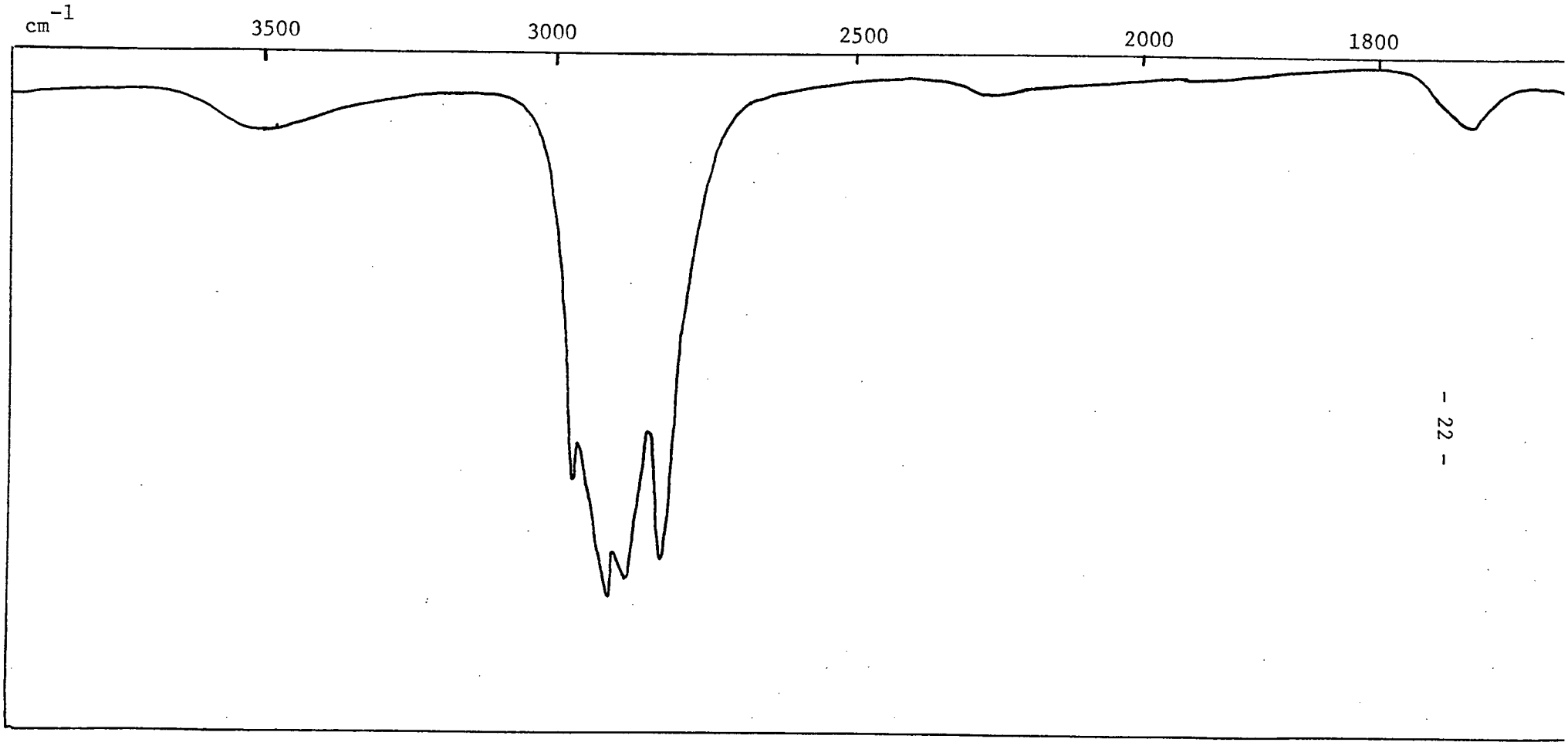


Figure 7. IR Spectrum of $SSGM_{15}^{302-371}$

methoxyl = 44.7%

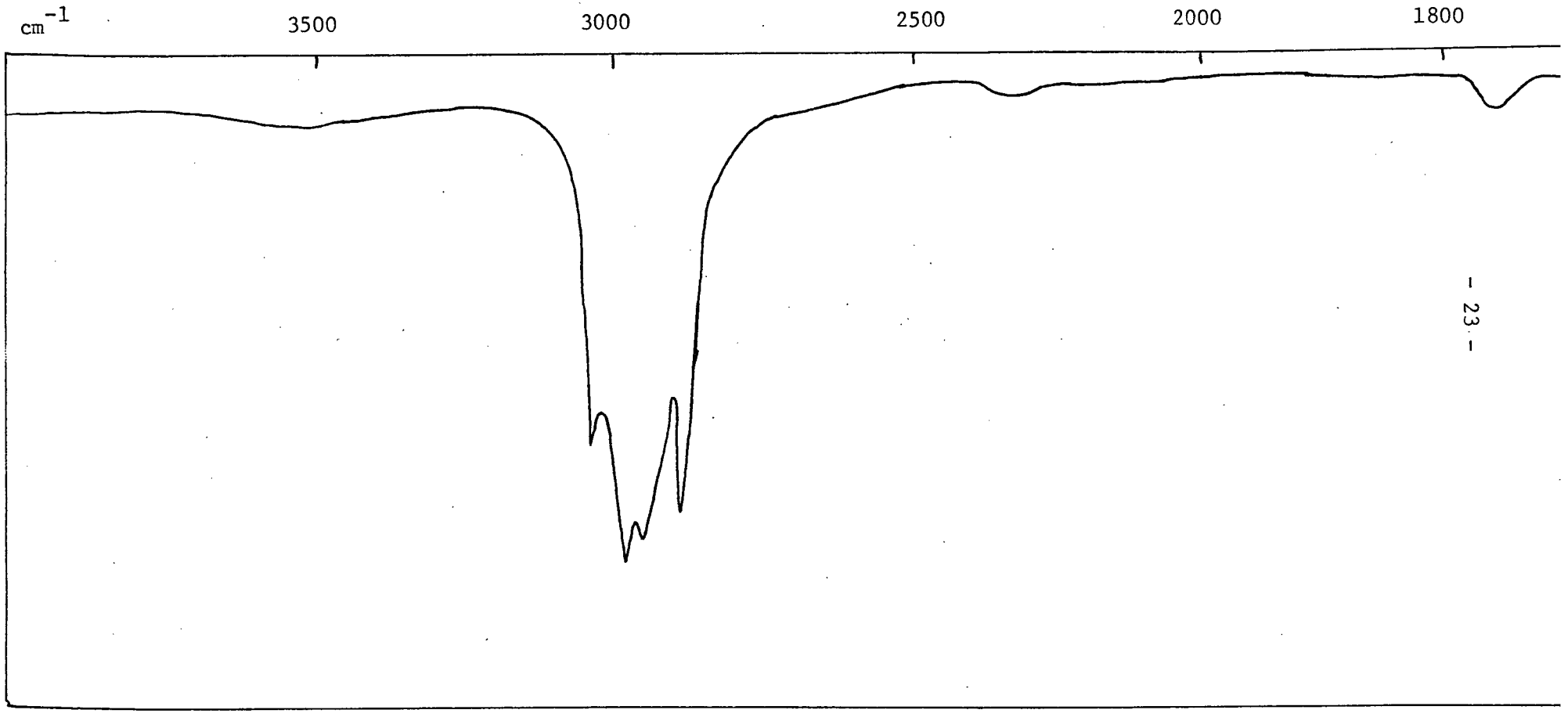


TABLE I. Descending Paper Chromatography of Methylated Sugars from SSGM and BSGM

MEK/H_2O Azeotrope

Black Spruce			Standards				Sitka Spruce		
Spot†	Rf. Obs.	Rel. Int.	Rf. Obs.		Rf. Lit.	Rf. Obs.	Spot†	Rf. Obs.	Rel. Int.
#1	.22	.10	.23	2,3-Me_2Man	.22	.19	#1	.17	0.10
-	?	-	-	2,6-Me_2Man	-	-	-	?	-
-	?	-	-	2,6-Me_2Gluc	.18	-	-	?	-
#2	.28	.04	.28	2,3-Me_2Gluc	.28	.23	#2	.22	.05
#3	.48	3.0	.50	2,3,6-Me_3Man	.50	.48	#3	.48	3.0
#4	.55	1.0	.56	2,3,6-Me_3Gluc	.56	.53	#4	.54	1.0
#5	.68	.03	.68	2,3,4,6-Me_4Gal	.68	.67	#5	.66	.02
#6	.78	.10	.78	2,3,4,6-Me_4Man	.78	.78	#6	.78	.10

Butanol/Ethanol/Water

Spot†	Rg. Obs.	Rel. Int.	Rg. Obs.		Rg. Lit.	Rg. Obs.	Spot†	Rg. Obs.	Rel. Int.
#1	.60	.1	.60	2,3-Me_2Man	.54	.62	#1	.61	0.10
-	?	-	-	2,6-Me_2Man	-	-	-	-	-
-	?	-	-	2,6-Me_2Gluc	.51	-	-	?	-
#2	.64	.03	.64	2,3-Me_2Gluc	.57	.68	#7	.67	.04
#3/4	.84	4.0	.84	2,3,6-Me_3Man and Gluc	.81 .83	.84	#3/4	.85	4.0
#5	.88	.03	.89	2,3,4,6-Me_4Gal	.88	.92	#5	.93	.05
#6	1.00	0.10	1.00	2,3,4,6-Me_4Man	1.00	1.00	#6	1.00	0.10

† The number identifies the spot on the paper chromatogram. The fastest moving component is #6.

TABLE II. Columns and Conditions used for G.L.C.

Derivative	Column	Packing	Flow Rate	Program	Comments
Alditol acetate	4' x 3/16"	3% ECNSS-M on 60-80 mesh Chrom.W or 100-120 mesh Gas Chrom.Q	100 ml/min	10' at 170° to 190° at 2°/min	Chromosorb W (non-silylated) gives longer retention time but improved resolution
Methylated alditol acetate	8' x 3/16"	"	86 ml/min	"	Column life is reduced if used over 195°
Persilyl ethers	8' x 1/4"	10% SF 96 on 60-80 mesh Diatoport S	86 ml/min	180-220° at 2°/min	
Methyl glycoside	10' x 1/4"	10% Apiezon N on 60-80 mesh Diatoport S	86 ml/min	175° isotherm.	Separates $MeMe_4$-α-mannoside from $MeMe_4$-α-glucoside
Methyl glycosides	8' x 1/4"	Carbowax 20 M on 60-80 mesh Diatoport S	86 ml/min	140°-210° at 2°/min	Separates $MeMe_4$-α-mannoside and α-glucoside from $MeMe_4$-α-galactoside
Methylated aldose acetate	8' x 3/10"	3% ECNSS-M on 100-120 mesh Gas Chrom Q	86 ml/min	a. 170° isotherm. b. 185° isotherm.	The lower temperature isothermal condition was used for separating Me_4 acetates

eight hours with 4 l. of water, allowed to stand eighteen hours and filtered. The residue was washed with 2 x 400 ml water and the combined filtrate and washings concentrated to 150 ml and poured into 600 ml alcohol. A flocculent precipitate formed which was centrifuged off, redissolved in a small volume of water and precipitated again. It was then dried by solvent exchange to give Sitka spruce water soluble polysaccharide (SSWSP) in a yield of 1.63 g or 0.33%.

3. Delignification of extracted wood.- Water extracted sawdust (200 g) was stirred with 3 l. of water and brought to 80°C. A total of 331 g of sodium chlorite and 92 ml of glacial acetic acid was added in four portions at intervals of one hour with continuous stirring. 2-Octanol was added dropwise as needed to reduce foaming. The extracted sawdust was washed with water until the washings were neutral and then twice with alcohol. A yield of 145 g was obtained. The procedure was repeated on a second lot of 200 g. The recovered Sitka spruce holocellulose was 142 g or 70% based on the weight of the dry wood.

4. Extraction of Sitka spruce holocellulose.- Holocellulose (280 g) was shaken with 2800 ml of 24% (w/w) potassium hydroxide solution for four hours, allowed to stand for ten hours and shaken for a further four hours. It was then filtered and washed twice with 400 ml water. The filtrate and washings were combined, neutralized with acetic acid and poured into alcohol. Drying by solvent exchange gave the product in a yield of 59 g equaling 21% of the holocellulose or 14.7% of dry wood.

5. Extraction of Sitka spruce glucomannan.- The extracted holocellulose (above) was washed thoroughly with water and then shaken for six hours with 2000 ml of 17% sodium hydroxide/3% boric acid solution. It was then permitted to stand for twelve hours, shaken for two hours and filtered. The residue was washed twice with 500 ml of water and the combined washings and filtrate were poured into alcohol/acetic acid. The precipitate was recovered by centrifugation and worked up by solvent exchange. Weight of crude SSGM was 72.8 g. Ash content was 27%. Corrected weight of SSGM was 53 g or 13% of dry wood.

6. Purification of Sitka spruce glucomannan.- Crude SSGM (45 g) was stirred for twenty-four hours with 500 ml of 17% sodium hydroxide/3% boric acid solution. As a large portion of the material appeared to remain undissolved the extraction was repeated with 300 ml fresh solvent for a further twenty-four hours. The combined suspensions were centrifuged; the centrifugate is SSGM Residue 1. The supernatant was precipitated with 500 ml 5% barium hydroxide solution added dropwise with constant stirring. The precipitate was washed twice with 25 ml 5% sodium hydroxide solution and then stirred at 0°C with 150 ml 50% acetic acid. The insoluble material was re-extracted with a further portion of 50 ml of 50% acetic acid. The combined solutions were precipitated into alcohol/acetic acid and worked up by solvent exchange. Yield of $SSGM_1$ = 23 g or 7% of dry wood.

The $SSGM_1$ was stirred with 220 ml 17% sodium hydroxide/3% boric acid for twelve hours and centrifuged. The residue was suspended

in 100 ml 50% acetic acid and stirred until no more material would dissolve. The mixture was then centrifuged and the centrifugate was termed SSGM Residue 2. The supernatant was poured into alcohol/acetic acid and recovered by solvent exchange. The recovered material was then dissolved by stirring for 4 hrs in 100 ml 17% sodium hydroxide/ 3% boric acid. The borate solutions were then combined and precipitated with 200 ml of 5% barium hydroxide. The precipitate was dissolved in 150 ml 50% acetic acid, precipitated into alcohol/acetic acid and worked up in the normal manner. Yield of $SSGM_2$ = 13 g or 4% of dry wood. This $SSGM_2$ was used for further methylation studies. The complete isolation scheme may be seen on the flow diagrams, Figs. 2 and 3.

7. Isolation of the black spruce.- The procedures followed were essentially identical with those just described for the Sitka spruce. The black spruce wood was not extracted for water soluble polysaccharides. Comparative yields and sugar ratios are given in Tables III and IV.

8. Hydrolysis of glucomannans.- The isolated polysaccharides were first solubilized by trituration in 72% sulfuric acid at 0°C for about one hour. The solution was then diluted to 1.0 N and hydrolyzed in a sealed tube at 98°C for eight hours. The solution was neutralized with barium carbonate and centrifuged. The solids were then extracted with water and centrifuged. This procedure was repeated three times. The combined centrifugates were passed through a short column of IR 120 and concentrated to a small volume. Paper chromatograms were run on this solution to detect the sugars present.

9. Preparation of derivatives for gas-liquid chromatography.- The trimethylsilyl derivatives were prepared by dissolving 10-20 mg of the sugar mixture, immediately after concentration just to dryness, in 2 ml of dry pyridine. Hexamethyldisilizane (HMDS) (0.5 ml) and chlorotrimethylsilane (TMS) (0.25 ml) were then added and the mixture allowed to stand five minutes.

Alditol acetates were prepared by dissolving a small portion of the sugar mixture in 15 ml of water and adding a solution containing a one molar excess of sodium borohydride. After 4-6 hours the excess borohydride was neutralized by the addition of 10% acetic acid. The solution was then deionized with IR 120 and borate salts were removed by evaporation to dryness several times with methanol.

The alditols were dissolved in 2 ml of a 50:50 mixture of acetic anhydride and pyridine and heated in a sealed tube at 98°C for 10 minutes. The acetylation reagents could then be removed on a vacuum rotary evaporator at 45-50°C as the alditol acetates are insufficiently volatile to be lost under these conditions. The mixture was then dissolved in a small volume of chloroform for injection on the g.l.c.

10. Hakomori methylation of Sitka spruce glucomannan.- The $SSGM_2$ (1.5 g) was passed through a 200 mesh sieve and dried at 60° for four hours under vacuum and placed in a flask fitted with a rubber serum cap. Dry nitrogen was passed through the flask after adding 75 ml of dry (distilled from calcium hydride) DMSO. Flask and contents were stirred (magnetic stirrer) for twenty-four hours.

TABLE III. Isolation of the Glucomannans

	Air dried wood	Oven dried wood	Benzene/ ethanol extracted wood	Delign-ified wood	KOH** extract	Crude** GM
Black Spruce						
(i) weight found	500 g	467 g		350 g	84 g	71 g
(ii) percent yield*				75	18.3	15.3
Sitka Spruce						
(i) weight found	500 g	465 g	455 g	344 g	73 g	66 g
(ii) percent yield*				74	15.7	14.2

* Based on weight of oven dry wood = 100%. ** Samples contain 18-25% ash.

TABLE IV. Percentage Composition of Isolated Polysaccharides.

	Percent** Yield	Man-nose	Gluc-ose	Galac-tose	Arab-inose	Xylose	$[\alpha]_D$ 1 N NaOH	$[\alpha]_D$ 17% NaOH 3% Borate
$SSGM_1$*	7.3	4.0	1.0	0.1	0.0	Tr		
$SSGM_2$	4.0	81.5	16.5	2.0	0.0	Tr	-27°	-40°
SSGM Res_1	0.5	84.0		16.0		Tr		
SSGM Res_2*	0.2	5.0	1.0					
$BSGM_2$	8.1							
$BSGM_2$*	2.7	4.0	1.0	0.1	0.0	Tr		-44.5°
$BSGM_2$ DMSO sol.	0.1							
$BSGM_2$ H_2O sol.	2.0	80.5	18.0	2.0	0.0	Tr	-31°	-43°
$BSGM_2$ insol.	0.5							-57°
BSGM Res 1	1.0							
BSGM Res 2	0.25	87.0	13.0					

* Ratios estimated from paper chromatography.

** Calculated on an ash free basis.

Sodium hydride (50% oil dispersion) was washed free of oil with dry 30-60° petroleum ether and allowed to react fully with DMSO (15 ml) first at room temperature for two hours then for four hours at 55°. An aliquot of DMSO-dimsyl ion solution was then removed and titrated with 0.1 N HCl after adding to 25 ml of water containing two drops of phenolphthalein. The amount of active sodium hydride was then calculated. Bottles of sodium hydride that had been some time in use were found to contain much less than the expected amount of active reagent. Sufficient sodium hydride (1.20 g) to provide a 100% excess calculated on the basis of three reacting hydroxyl functions per sugar residue was then reacted in the same manner with 25 ml DMSO. The dimsyl ion, which formed a clear brown-green solution, was then added to the polysaccharide by injecting through the rubber serum cap with a hypodermic syringe. There was immediate and extensive gel formation and the mixture turned a dark brown colour. After four hours a 100% excess* of methyl iodide was then added (7.1 g ≡ 3.1 ml) in increments of 0.25 ml over six hours with constant stirring. The temperature of the reaction was not allowed to rise above 15°C. The mixture was then light straw in colour and still slightly cloudy indicating incomplete solution. It was stirred for a further 12 hrs and then diluted with 4 volumes of cold

---

* Based on the reaction:

$$Na^{+} \; {}^{-}CH_2\text{-}\overset{\overset{O}{\|}}{S}\text{-}CH_3 + CH_3I \longrightarrow CH_3\text{-}CH_2\text{-}\overset{\overset{O}{\|}}{S}\text{-}CH_3 + NaI$$

water and dialyzed against running water for 72 hrs. The polysaccharide was then extracted with refluxing chloroform and a methoxyl analysis was done on the crude extract. OMe = 28.2%.

The methylation procedure was then repeated on the partly methylated polysaccharide after drying it under vacuum. The polysaccharide was dissolved in 50 ml DMSO and 1.0 g NaH (as dimsyl ion) and 2.5 ml methyl iodide were added. The methylated polysaccharide was recovered as before in a yield of 1.36 g. The material was then fractionally extracted with chloroform/petroleum ether (30-60°) solvents. The samples $SSGM_{15}^{308}$ and $SSGM_{20}^{368}$ were used for gas-liquid chromatography. A portion (165 mg) of the fraction 302-308 was remethylated using Purdie's reagents. The sample was dissolved in 25 ml methyl iodide and 7 g of freshly prepared silver oxide was added at intervals to the refluxing solution over a period of seventy-two hours. The solution was then centrifuged to remove the bulk of the silver salts which were then extracted for twenty-four hours in a Soxhlet extractor with chloroform. The solutions were combined, concentrated to dryness and fractionally extracted with chloroform/petroleum ether as for the crude methylated polysaccharide. This procedure effectively separates the polysaccharide from colloidal silver salts. The data for the material recovered in the 15% chloroform solvent is included in Table V. It was hoped to demonstrate with this more highly methylated polymer that the last peak in the gas-liquid chromatograph of methylated sugars was derived from sugars present as a result of undermethylation. The gas-liquid chromatography results obtained may be seen in Table VIII and their significance is discussed in the interpretation.

11. Methylation of the black spruce glucomannan.- Initially 1.31 g of $BSGM_2$ was taken for methylation and stirred for forty-eight hours at 50° in 60 ml of dry DMSO. As only a small amount was observed to dissolve the insoluble material was removed by centrifugation. A portion of the insoluble material was stirred with water for twenty-four hours and all samples were dialyzed for twenty-four hours and recovered by freeze drying. This gave three fractions as shown in Table VI. Methylation was carried out on 694 mg of the water soluble $BSGM_2$ as described for the $SSGM_2$ using 1.75 g of NaH (free of oil). The polysaccharide isolated after dialysis was only partially methylated and was remethylated using 1.5 g NaH in 50 ml DMSO. The material was then dialysed and subjected to fractional extraction with petroleum ether/chloroform after removal of water on the rotary evaporator.

As the methoxyl value for the combined 10 and 15% extracts was deemed to be only marginally high enough they were remethylated using 0.25 g NaH. Little gel formation was seen to occur in this instance. The product was isolated in the customary manner and analysed. As the methoxyl value had increased only very slightly this sample was used for subsequent analyses.

12. Hydrolysis of the methylated polysaccharides.- The importance of minimizing degradation, demethylation and loss of volatile methylated sugars has been emphasized in the discussion. Individual methylated polysaccharides were hydrolysed by either method as

described below and listed in Table VII.

13. Hydrolysis with 90% formic acid.- $SSGM^{302-308}$ (150 mg) was placed in a small round bottomed flask with 10 ml of 90% formic acid and refluxed for one hour. The formic acid was then removed at 40°C on a rotary evaporator and 10 ml of 1 N sulfuric acid was added and refluxed for six hours. The sulfuric acid was then neutralized with a slurry of barium carbonate. The suspension was centrifuged and the precipitate extracted with water and centrifuged. This procedure was repeated three times. The centrifugates were deionized with IR 120 and then extracted with chloroform to remove the more highly methylated and, therefore, more volatile sugars. The aqueous mixture was then evaporated to dryness at 40°; the chloroform extract was added and evaporated at 30°C.

14. Hydrolysis with 72% sulfuric acid.- $BSGM_{20}$ (5 mg) was mixed with 0.5 ml of 72% sulfuric acid and held at 0°C for one hour or until the polysaccharide was observed to be completely in solution. The solution was then diluted to 10 ml and refluxed for six hours. Work up then followed the procedure outlined above. It is useful, perhaps, to note also that such a 5 mg sample upon reduction and acetylation provided just sufficient material for two injections on our gas-liquid chromatograph of sufficient size to

TABLE V. Fractionation of Methylated SSGM

Solvent (% $CHCl_3$)	Weight	OMe	Ash	OMe*	$[\alpha]_D^{25}$ $CHCl_3$	Anal.#
5	.021					
10	.397				-25.1°	302-309
15	.721	43.6	1.78	44.4	-26.6°	302-308
20	.187	39.1			-23.7°	302-368
30	.010					
302-308 remethylated	.150	44.7	0.00	44.7	-23.8°	302-371

TABLE VI. Fractionation of Methylated BSGM

Solvent (% $CHCl_3$)	Weight	OMe	Ash	OMe*	$[\alpha]_D^{25}$ $CHCl_3$	Anal.#
10	.124	43.1	1.6	43.9	-17.4°	302-298
15	.376				-19.4	
20	.397				-23.9	
25	.031					
30	.023					
40	.007					
302-298 remethylated	.300	43.66	1.11	44.1		302-302

* The ash in these samples may be accounted for in two ways (see p. 19): either as an inert non-volatile contaminant or as the residue derived from cations originally combined with uronic acids. As no traces of uronic acid were found in hydrolysates of the methylated polysaccharides the former case was assumed.

TABLE VII. Methods used for Hydrolysis of Individual Methylated Polysaccharides

Polysaccharide	Anal. #	Hydrolysis Method
$SSGM_{15}$	302-308	Formic acid ⟶ H_2SO_4 1 N
$SSGM_{15}$	302-371	72% Sulfuric ⟶ H_2SO_4 1 N
$SSGM_{20}$	302-368	Formic acid ⟶ H_2SO_4 1 N
$BSGM_{10-15}$	302-298	Formic acid ⟶ H_2SO_4 1 N
$BSGM_{20}$ [a]		Formic acid ⟶ H_2SO_4 1 N
$BSGM_{20}$ [b]		72% Sulfuric ⟶ H_2SO_4 1 N

integrate peaks greater than or equal to about 0.5% of the total.

15. Reduction of methylated sugars.- All reductions were carried out in aqueous solution with sodium borohydride. The mixture of methylated sugars was dissolved in a small volume of water and a solution containing a one molar excess of sodium borohydride was added and allowed to stand overnight. Excess borohydride was then neutralized by the addition of 10% acetic acid until evolution of hydrogen ceased. The solution was then passed through a column of IR 120 ion exchange resin and concentrated to dryness. The mixture of reduced sugars and salts was then evaporated three times with methanol to remove boric acid as the volatile methyl borate (51).

16. Acetylation of methylated alditols or aldoses.- The sugars were removed from the flask by dissolution three times in a small volume of a 50:50 mixture of acetic anhydride/pyridine (total 8 ml) and placed in a test tube which was sealed and heated at 98° for 15 minutes. By this method acetylation was found to proceed invariably to completion. The acetylation was attempted once in an unsealed vessel on top of a steam bath but was found to fail to go to completion as was shown by continued presence of an OH peak in the IR spectrum of the product. Alditol acetates were extracted from the acetylation mixture by the following procedure. Extraction three times in a separatory funnel with chloroform, extraction twice of the chloroform extract with 1 N HCl, washing once with sodium bicarbonate and once with water. The chloroform solution was then dried over calcium chloride and concentrated to a suitable volume for injection on the

gas-liquid chromatograph. If this extraction was not performed anomalous peaks were found in chromatograms of samples that had remained in the acetylation mixture for some time.

17. Paper chromatography.- Paper chromatography was done both on free sugars from the unmethylated polysaccharide and on methylated sugars. The former were run using the eluents 8:2:2 ethyl acetate/pyridine/water or 18:3:1:4 ethyl acetate/acetic acid/formic acid/water. The first solvent is a basic medium and will resolve glucose, galactose, mannose, arabinose, and xylose in twenty-four hours. The second solvent is acidic in nature and is used to separate acidic sugars. Similarly for methylated sugars two eluents were routinely employed. Methyl ethyl ketone/water azeotrope for one front time (approximately four and one-half hours for 70 cm paper) or butanol/ethanol/water 4:1:3 for twenty-four hours. The former was found to be most useful when attention was directed toward the faster moving tetramethyl hexoses and trimethyl pentoses, the latter gave superior separation of dimethyl hexoses. Chromatograms of free sugars were developed by dipping in (1) silver nitrate, (2) sodium hydroxide, (3) sodium thiosulfate. Methylated sugars were developed with p-anisidine spray followed by heating at 118° for five minutes. The Rf. and Rg. values observed for sugars from polysaccharides, standard samples and recorded literature values are listed in Table I (49,50).

18. Preparative paper chromatography.- About 100 mg of the mixture of methylated polysaccharide hydrolysates of Sitka spruce and black spruce were streaked on four sheets (25 x 70 cm) of Whatman No. 1 chromatography paper and developed in methyl ethyl ketone/water. After drying, guide strips were cut from the sides

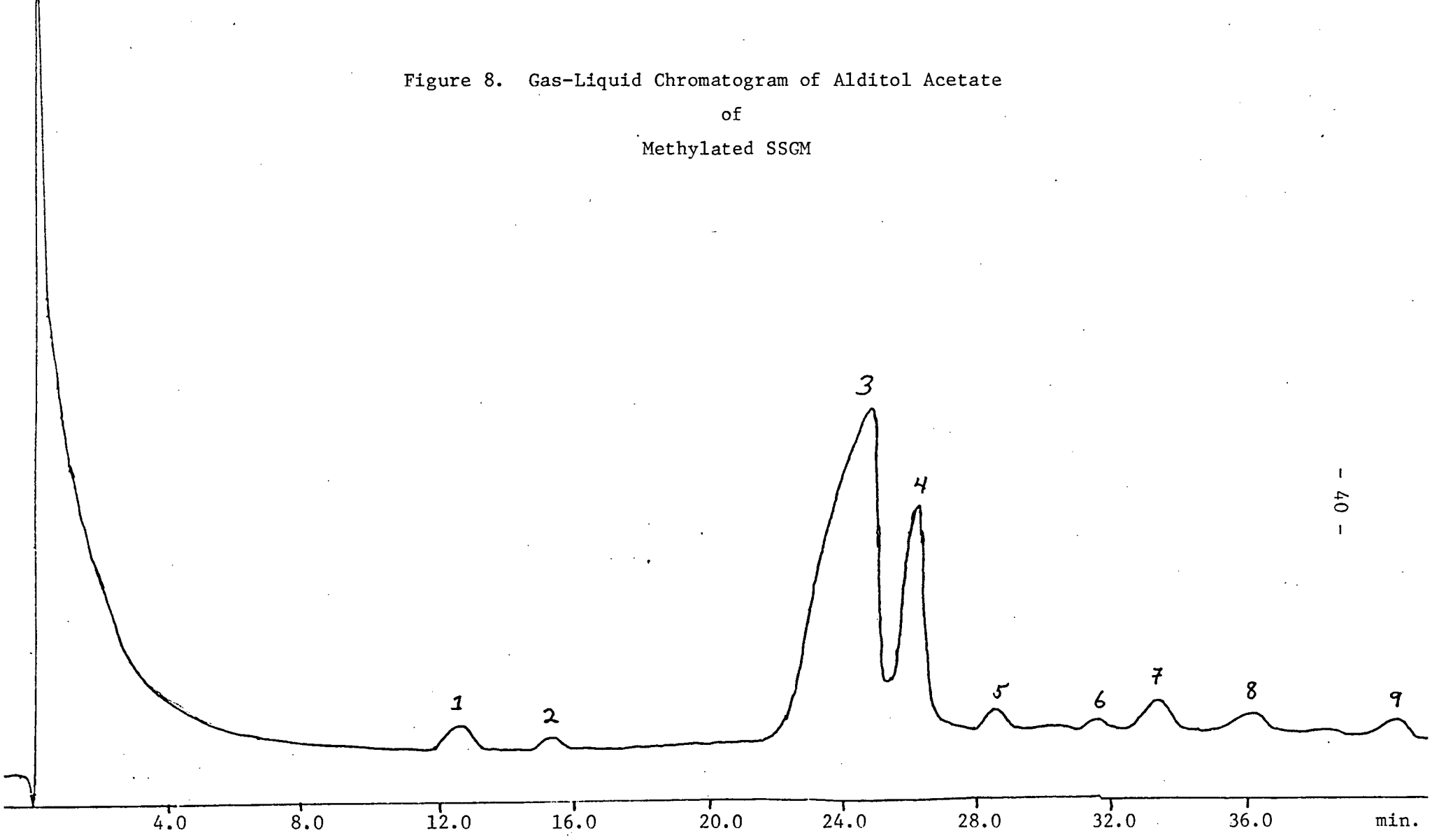
of the papers to determine the position of the bands. Four strips were cut corresponding to those spots designated 1 and 2, 3 and 4, 5 and 6 in Table I. Methylated sugars were removed from the strips by eluting with water and then concentrated to dryness at low temperature.

19. Gas-liquid chromatography.- Samples were prepared for gas-liquid chromatography in solution in either chloroform or acetone for alditol acetates, methylated alditol acetates, methylated sugar acetates and methyl glycosides. Silyl derivatives were injected directly or evaporated to dryness and dissolved in hexane. Systems and conditions used for various derivatives are listed in Table II. Sample size was determined by the information desired. Small samples were chromatographed to determine relative ratios of major components. Larger sample size was used so that the peaks of minor components appeared at sufficient intensity to activate the integration circuits and provide reliable measurement. Sample chromatograms are reproduced in Figs. 8 and 9.

20. Preparation of methyl glycosides.- Methyl glycosides were prepared from methyl sugars isolated from preparative paper chromatography of methylated BSGM and SSGM hydrolysates. Sugars were dissolved in 25 ml 3% hydrogen chloride/methanol and refluxed for eight hours. Previous work (52,53) had shown that this produced essentially complete equilibration of the isomers formed. Hydrochloric acid was neutralized with silver carbonate and after filtration the glycosides were concentrated to dryness at 35°C.

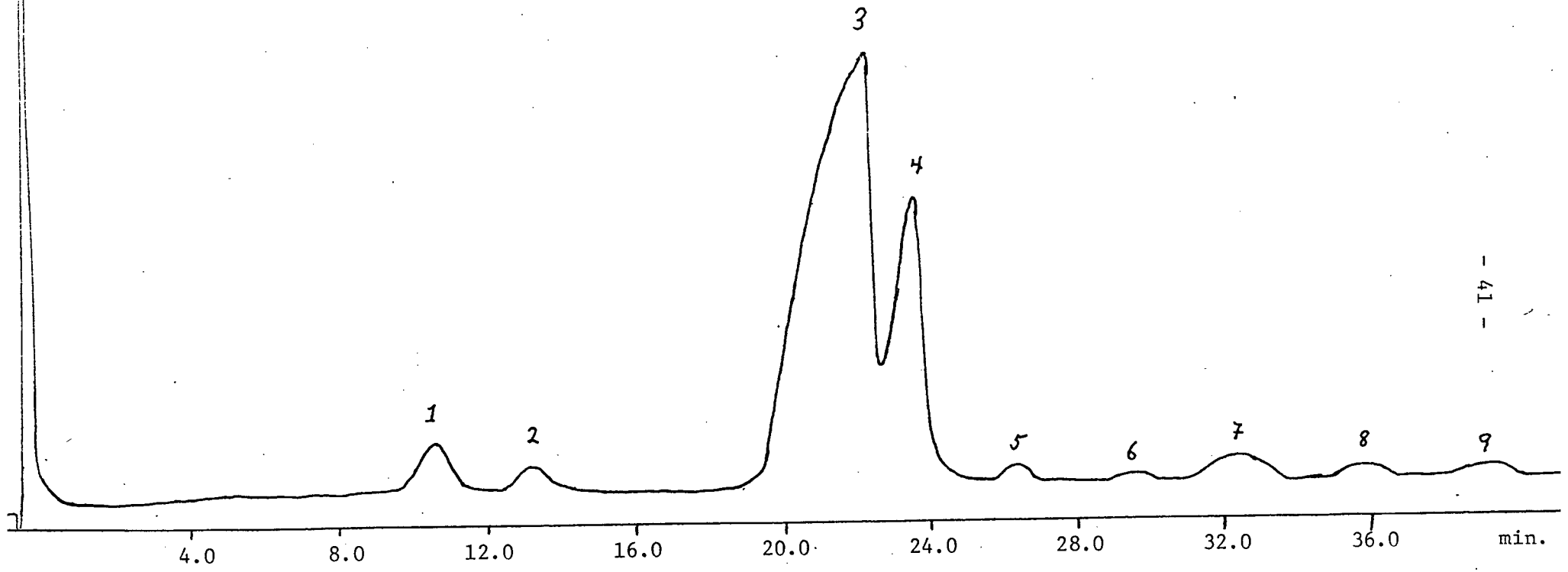
21. Mass spectroscopy.- Samples were collected from the gas-liquid chromatography of methylated alditol acetates for mass spectro-

Figure 8. Gas-Liquid Chromatogram of Alditol Acetate
of
Methylated SSGM



3% ECNSS-M on Chromosorb W. 10 min. at 170° to 190° at 2°/min. Flow rate = 86 ml/min.

Figure 9. Gas-Liquid Chromatogram of Alditol Acetate
of
Methylated BSGM



3% ECNSS-M on Chromosorb W. 10 min at 170° to 190° at 2°/min. Flow rate = 86 ml/min.

TABLE VIII. Amounts* of Methyl Alditol Acetates as Determined by G.L.C.

Polysaccharide	#1 Me_4M	#2 Me_4Gal	#2' Me_2X	#3 Me_3M	#4 Me_3G	#5 Me_2M	#6 Me_2G	#7 Me_2M	#8 Me_2G	#9 Me?
$BSGM_{15}$	2.77	1.33	-	76.6	17.3	0.65	tr.	0.80	0.70	tr.
$BSGM_x$	1.6	0.5	0.1	78.0	18.5	0.25	tr.	1.5	0.3	tr.
$BSGM_{20}$ [a]	0.45	2.0	1.8	71.6	21.5	0.2	0.3	2.2	0.5	0.4
$BSGM_{20}$ [b]	1.15	1.65	2.6	67.8	20.4	1.66	1.04	4.65	0.8	0.7
$BSGM_{20}$ comb.	1.15	1.65	2.6	70.2	20.9	0.2	0.3	2.2	0.5	0.4
$SSGM_{15}$ [a]	1.7	0.3	-	76.6	17.2	0.6	0.2	1.5	0.5	0.7
$SSGM_{15}$ [b]	3.12	0.92	-	71.5	17.6	1.8	1.15	2.17	0.7	0.9
$SSGM_{15}$ comb.	3.02	0.90	-	75.3	16.8	0.6	0.2	1.5	0.5	0.7
$SSGM_{20}$	0.72	0.23	~0.3	67.8	21.4	1.47	1.55	3.7	1.04	1.7

* Expressed in terms of mass percent of the total.

TABLE IX. Ratios of Methylated Sugars Found in the Polysaccharides

Methylated polysaccharide	Ratio of total methyl mannose/methyl glucoses	Me_4 galactose (%)	Tetra methyl sugars (%)	dimethyl sugars (%)	2,3-dimethyl sugars (%)	δ	δ'
$BSGM_{15}$	4.50	1.33	4.13	2.15	1.50	38	51
$BSGM_{20}$ [a]	3.34	1.65					
$BSGM_{20}$ [b]	3.32	1.80					
$BSGM_{20}$ comb.	3.32	1.70	2.80	3.20	2.70		
$SSGM_{15}$ [a]	4.45	0.30					
$SSGM_{15}$ [b]	4.05	0.92					
$SSGM_{15}$ comb.	4.45	0.92	3.92	2.90	2.00	52	97
$SSGM_{20}$	3.10	0.23	0.95	7.76	4.74		

$$\delta = \frac{100}{\%\ Me_4\ \text{sugars} - \%\ 2,3\text{-dimethyl sugars}}$$

$$\delta' = \frac{100}{\%\ \text{tetramethyl sugars} - \%\ \text{total dimethyl sugars}}$$

The δ value as defined above should indicate the degree of polymerization in the polymer if only 2,3-di-O-methyl hexoses are structurally significant (i.e. if 2,6-di-O-methyl hexoses result from under- and/or demethylation). If all dimethyl sugars are significant the δ' value should correspond to the chain length. Similarly the percentage of branch points is indicated by the percentage of 2,3-di-O-methyl hexoses or of all dimethyl hexoses.

metric analysis and for demethylation. Samples were run on either an A.E.I. MS 902 instrument or a Nuclide 1290 G. Ionizing voltages and source temperatures were 70 Ev and 150°C, respectively. The results obtained are discussed in the following section. Proposed explanations of the fractionation patterns may be found in the literature (42,43,54). They are not further discussed here as the mass spectrometry was employed only as a means of qualitative identification of the methyl alditols. A typical simplified mass spectrum is shown in Figure 10.

22. Demethylation.- Samples of methyl alditol acetates for demethylation were washed from the capilliary tubes in which they were collected with 2 ml of dichloromethane and cooled in small flasks to -78°C (dry ice/acetone) (48). About 1 ml of boron trichloride was then added; the mixtures were maintained at -78°C for one hour and then allowed to warm to room temperature and left standing for sixteen hours. They were then evaporated three times with methanol and acetylated by the procedure previously described. Demethylation was generally complete although occasionally small amounts of partially methylated alditol acetates could be detected upon subsequent gas chromatography. Methylated mannitols appeared to be particularly resistant to complete demethylation.

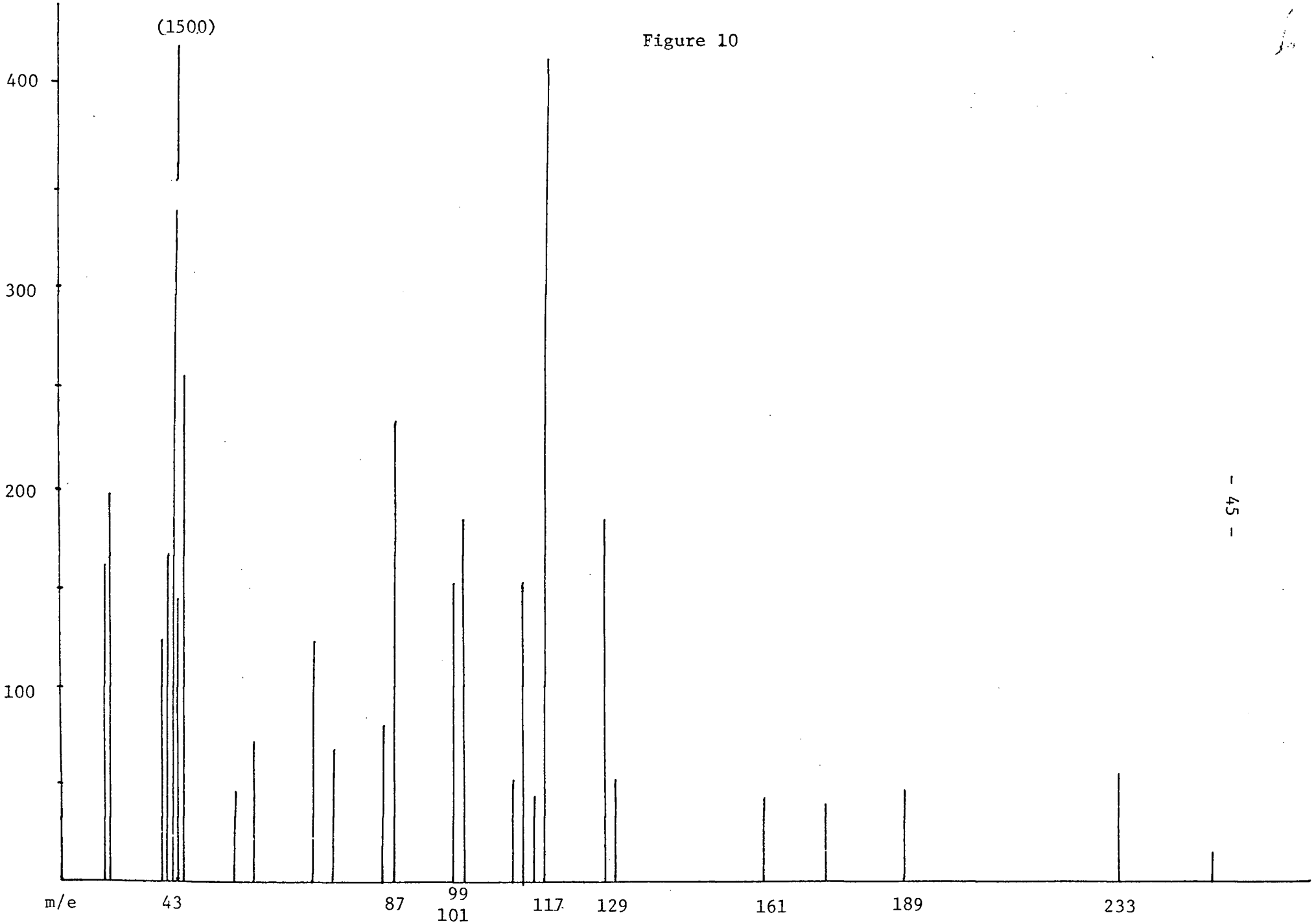


Figure 10

Simplified Mass Spectrum of SSGM peak #6 2,3-di-O-methyl-D-glucitol tetraacetate.

RESULTS AND INTERPRETATION

The summary of data and its interpretation may be conveniently divided into two parts. The first dealing with the establishment of the identity of methylated alditols obtained and the second a discussion of structural features of the polysaccharides which may be inferred.

For convenience of reference the peaks observed in chromatograms of the methylated alditol acetates have been designated 1-9. The same nine peaks are observed for both glucomannans with only the percentages present varying slightly.

Identification of methyl sugars

Peak #1.- This had the mass spectrum of a 2,3,4,6-tetra-O-methyl-hexitol, showing the following peaks in high intensity: m/e; 43, 45, 71, 87, 101, 117, 129, 145, 161, and 205. The peak had the same retention time as 2,3,4,6-tetra-O-methyl-D-glucitol and -mannitol acetates. On paper chromatography a spot with the retention time of 2,3,4,6-tetra-O-methyl-mannose (or glucose) was observed. Isolation of this and preparation of the methyl glycoside provided the following results.

(i) the glycoside did not co-chromatograph with methyl 2,3,4,6-tetra-O-methyl-α-D-galactoside on Carbowax 20 M and gave a peak

with the same retention times as methyl 2,3,4,6-tetra-O-methyl-α-D-mannoside or -glucoside (52). No peak corresponding to methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside was observed (if the sugar was glucose some of the β-anomer should be formed on methanolysis).

(ii) On Apiezon N the glycoside co-chromatographed with methyl 2,3,4,6-tetra-O-methyl-α-D-mannoside and not with the corresponding α-D-glucoside.

(iii) Additional confirmation was obtained for the BSGM by g.l.c. of the acetates of the methylated aldoses. The relative retention times observed for some standard samples are reported in Table X. In the chromatogram of BSGM methylated sugar acetate peaks with retention times corresponding to those of 2,3,4,6-tetra-O-methyl-D-mannose and galactose acetates were found.

It may be concluded that peak #1 corresponds to 2,3,4,6-tetra-O-methyl-D-mannitol and that no tetra-O-methyl-D-glucitol is present within the limit of detection (about 1/5 of the amount of tetramethyl mannitol found).

Peak #2.- This has also the mass spectrum expected for a 2,3,4,6-tetra-O-methyl-D-alditol acetate and the same retention time as 2,3,4,6-tetra-O-methyl-D-galactitol. On paper chromatography a faint spot with the retantion time of 2,3,4,6-tetra-O-methyl-D-galactose is observed. The gas chromatography of the methyl glycoside showed:

(i) One peak on Carbowax 20 M with the retention times of methyl 2,3,4,6-tetra-O-methyl-α + β-D-galactosides and nothing with the retention times of the corresponding α-D-glucosides of α-D-mannosides.

(ii) When chromatographed on Apiezon N the glycoside gave two

TABLE X. Retention Times of Methylated Aldose Acetates

Compound	Ret. time	Ret. time	Approx. Ratio
2,3,4,6-tetramethyl glucose acetate	9.4	11.5	.85
2,3,4,6-tetramethyl galactose acetate	13.4	20.2	1.4
2,3,5,6-tetramethyl galactose acetate	15.1		
2,3,4,6-tetramethyl mannose acetate	17.9	23.3	20
Conditions: Isothermal at 170°C			
2,3,4,6-tetramethyl glucose acetate	4.8	5.2	
2,3,6-trimethyl glucose acetate	14.8	17.0	1.3
2,3,6-trimethyl mannose acetate	31.2		
2,3-dimethyl glucose acetate	34.8	39.7	.3
Conditions: Isothermal at 185°C			

peaks with the retention times of methyl 2,3,4,6-tetra-O-methyl-α and β-D-galactosides.

Peak #2 is therefore 2,3,4,6-tetra-O-methyl-D-galactitol.

Peak #3.- As the largest peak in the chromatograms this compound should be 2,3,6-tri-O-methyl-D-mannitol acetate (60). The mass spectrum for it exhibited the major fragments: m/e; 43,45, 87, 99, 101, 113, 117, and 233 as expected for a 2,3,6-tri-O-methyl-D-alditol acetate. The retention time on g.l.c. was the same as that for 2,3,6-tri-O-methyl-D-mannitol acetate and the Rf. and Rg. values on paper chromatography were the same. Finally, upon demethylation and reacetylation only mannitol hexaacetate was found when the product was gas chromatographed on a column of ECNSS-M. M.p. 118°C. Lit. value 123°C.

Peak #4.- As the second major component this peak should be 2,3,6-tri-O-methyl-D-glucitol acetate. The mass spectrum, major fragments: m/e; 43, 45, 87, 99, 101, 113, 117, and 233, is consistent only with a 2,3,6-tri-O-methyl-D-hexitol acetate. Paper chromatography in both solvents showed spots with Rf's exactly corresponding to those of authentic 2,3,6-tri-O-methyl-D-glucose. The hexitol acetate derived after demethylation was glucitol with only traces of mannitol present. M.p. 97°C. Lit. value 99°C.

Peak #5.- The mass spectrum of this peak showed major fragments: m/e; 45, 87, 117 and 129 which is consistent only with a 2,6-di-O-methyl-D-hexitol acetate. The retention time on g.l.c. was close to that reported for 2,6-di-O-methyl-D-mannitol acetate (38) (no authentic sample was available). Mannitol acetate was obtained upon gas liquid

chromatography of the demethylated and acetylated material. The identity may be further substantiated by a process of elimination: 2,3-di-O-methyl-D-mannitol had been identified as peak #7, 2,4- and 3,4-di-O-methyl-D-mannitols have g.l.c. retention times as reported by Lindberg (38) that are very much too great and in addition their mass spectra are very different to that observed. 4,6-Di-O-methyl-D-mannitol was eliminated by the absence of intense fragments in the mass spectrum at m/e; 101, 161 and 261. 2,3,4-, 2,4,6-, and 3,4,6-tri-O-methyl-D-mannitols were eliminated as all have reported retention times less than that of 2,3,6-tri-O-methyl-D-glucitol. 2,3,5-Tri-O-methyl-D-mannitol was eliminated by the absence of any large amount of the fragment m/e 101, and the presence of fragments of m/e 45, 87, and 129.

Peak #6.- As may be seen from the figures in Table VIII only very small amounts of this peak were present. The mass spectrum was essentially identical to that described for peak #5. The g.l.c. retention time is the same as that of 2,6-di-O-methyl-D-glucitol acetate. After demethylation and reacetylation the presence of glucitol was strongly indicated but not confirmed because of the very small amount available.

Peak #7.- This peak showed the mass spectrum of a 2,3-di-O-methyl-D-hexitol with major fragments: m/e 43, 101, 117, and 261. It co-chromatographed with a standard sample of 2,3-di-O-methyl-D-mannitol acetate. A spot with Rf and Rg corresponding to that of 2,3-di-O-methyl-D-mannose was seen on paper chromatography and mannitol hexaacetate was found after demethylation and acetylation. M.p. 119°C. Lit. value 123°C.

Peak #8.- The mass spectrum of this peak was very similar to that of peak #7. Both fitted only the fragmentation pattern reported for a 2,3-di-O-methyl-D-hexitol although the intensity of peak 101 was rather low and that of 161 was somewhat higher than the values quoted by Lindberg. The same chracteristics were however also found when a mass spectrum was made of an authentic sample of 2,3-di-O-methyl-D-mannitol acetate. The differences are presumably due to changes in the instrumental parameters used in obtaining the spectra. The g.l.c. retention times of the substance was exactly the same as that of 2,3-di-O-methyl-D-glucitol acetate. On paper chromatography a spot slightly fainter than that for 2,3-di-O-methyl-D-mannose was observed with the same retention times as for authentic 2,3-di-O-methyl-D-glucose.

Peak #9.- The identity of this peak was not satisfactorily established. The g.l.c. retention time indicated that it probably was a monomethyl alditol acetate or a mixture of one or more. The mass spectrum was inconclusive as there was at least one significant fragment for either 2-, 3-, or 6-mono-O-methyl alditols which did not appear. The most probable choice is a 2-mono-O-methyl derivative as for this the only observed discrepancy between the observed fragmentation pattern and that reported is the absence of a fragment of m/e 139 (41). When the material was demethylated and re-chromatographed as the alditol acetate both mannitol and a small amount of galactitol were found. As it was thought that the compounds arose from under- (or de) methylation of the polysaccharide and its presence in very small percentage makes any structural significance unlikely no further

attempt was made to determine its identity.

Structural Significance of the data

The amounts of individual methylated sugars present and their significance may now be discussed. The results obtained are presented in Table VIII. The peak referred to as 2' has been identified by mass spectrometry etc. as 2,3-di-O-methyl-D-xylitol. It arises from a small amount of contaminant xylan in the original polysaccharide (16,55,56).

When the data were compiled it was observed that, where comparisons were possible, polysaccharides that had been solubilized in 72% sulfuric acid had a higher ratio of tetramethyl sugars than those that had been reacted with formic acid. At the same time those polysaccharides hydrolyzed in 72% sulfuric acid also had relatively higher amounts of dimethyl sugars. This can be clearly seen by referring to the values recorded for $SSGM_{15}$ (308) and $SSGM_{15}$ (371) the latter had a higher methoxyl value and yet contributed almost twice as much dimethyl hexose. It is only logical to explain these results by assuming that a small amount of tetramethyl sugar may be lost during the relatively long evaporation time required to remove the formic acid. Similarly it appears that a significant increase in demethylation takes place when 72% sulfuric acid is used. As no tetramethyl sugars can logically be present that were not an integral part of the original polysaccharide the maximum values obtained for them were assumed to be most valid. Similarly no dimethyl sugar should be lost in work up and minimum values (excess arising from demethylation) should be the most reliable. With this in mind the

amounts of tetramethyl sugars reported for $SSGM_{20}$ (which was done only by the formic acid method) are probably a little below the actual values.

On the whole the amount of structural information which may be derived from the results is disappointingly small. The problem revolves about the combined effects of very small amounts of dimethyl sugars (0.5-1.5%) which may or may not be significant, the problems of purity, and of complete methylation. The problem of under-methylation may be dealt with first. Our values of methoxyl content are within .7-1.4% of the theoretical and were not significantly raised upon remethylation. Unfortunately even a three fold improvement to about 0.25% (which is close to the limit of reproducibility of methoxyl analysis) would still permit that as much as .55% of all possible hydroxyl functions might remain unmethylated (35). This is equivalent to having one and one-half moles of dimethyl sugars for every 100 moles of the expected trimethyl sugar. Clearly, under such a limitation the presence of 0.5% of a particular dimethyl sugar cannot be used as a basis for structural determination.

The question of purity of polysaccharides introduces similar problems. It is certain from the detection of small amounts of 2,3-di-O-methyl-D-xylitol in some fractions of the methylated glucomannans that a trace of xylan remains in the polysaccharide after many purification steps. The presence of xylan is not in itself prejudicial to the analysis but it does indicate that one must expect the presence of some GGM which in solubility characteristics is much more akin to GM than is xylan. Having admitted this possibility one can no longer say with certainty that the tetramethyl galactitol or

galactose found in the GM is actually a part of it or is derived from a small amount of admixed GGM. In fact it is shown quite clearly that the glucomannan used for methylation analysis is not completely homogeneous, even if the small amount of admixed xylan is ignored. There is a marked difference in the ratios of mannose/glucose determined for the 15% and 20% chloroform/petroleum ether extracts of methylated polysaccharides. This difference 4.45:1 for $SSGM_{15}$ as compared to 3.10:1 for $SSGM_{20}$ is much greater than any species differentiation observed (Table IX). It is possible that no true GM or GGM can be defined for these softwood species and that instead a continuous series of polysaccharides of gradually increasing degree of branching and galactose content may present a more accurate picture. My own opinion, however, is that the solubility and precipitation characteristics of the GM's and GGM's are sufficiently different to indicate two distinct classes of polysaccharides and that the GM's do obtain a small percentage of galactose and have a low degree of branching (2, 57,58). This is not proven, however, but could be by rigorously establishing the purity of the initial polysaccharide. The remarks made above apply equally to the GM's of black and Sitka spruces. A brief summary may be made for the specific results obtained for each as in Table IX. All polysaccharides produce 1.5-3.5% 2,3-di-O-methyl-D-mannose which indicates that most branching occurs through the six position of the mannose residue. A small amount of branching probably occurs through the six position of glucose residues as 2,3-di-O-methyl-D-glucose is not likely to occur as an artifact of undermethylation since the six position is relatively easily methylated.

The small amounts of 2,6-di-O-methyl sugars obtained are probably derived from undermethylation. For the samples extracted in 15% chloroform/petroleum ether the amounts of tetramethyl and 2,3-di-O-methyl sugars suggest a chain length of about forty for BSGM and fifty for SSGM. It is likely that some degradation of the original polymer has occurred during the methylation or extraction (1,62). The ratios of the total methylated mannose to methylated glucose 4.45:1.00 for SSGM compares reasonably well to the ratios of about 4.8:1.00 for the unmethylated polysaccharide as does the amount of 2,3,4,6-tetra-O-methyl-D-galactitol (approximately 1%). The equivalent results for BSGM are mannose/glucose 4.4:1, methylated mannoses/methylated glucoses 4.5:1 and 1.3% 2,3,4,6-tetra-O-methyl-D-galactose.

In conclusion it may be said that the method of g.l.c. of the alditol acetates and combined mass spectrometry provides a relatively rapid and accurate means of analysing the methylated sugars derived from polysaccharides. It can be applied to very small quantities once the identity of components to be expected has been demonstrated. Thus, initial analysis could be done on a large, relatively impure sample and the structure refined by a study on a small amount rigorously purified, perhaps by electrophoretic techniques (28,29). In the present case, extremely high degrees of purity and completeness of methylation would have to be obtained to establish conclusively what are at most extremely minor structural features in the glucomannans. If, for example, one wishes to prove absolutely the existence of one branch point per 100 sugar residues, the level of possible

impurity (if of a nature which could contribute a species leading to misinterpretation) must be reduced to 0.5% or less and the methoxyl value should be within 0.05% of the theoretical. At present the latter criterion, even in view of the improved methylation techniqes cited earlier does not appear to be a practical possibility.

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