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Physical Properties and Chemical Composition of β-Glucans from Fleshy Fungi

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ABSTRACT

WALLEN, L. L. (Northern Regional Research Laboratory, Peoria, Ill.), R. A. RHODES, AND H. RUSSELL SHULKE. Physical properties and chemical composition of β-glucans from fleshy fungi. Appl. Microbiol. **13**:272–278. 1965.—Physical properties and chemical structure of two related polysaccharides produced fermentatively by *Plectania occidentalis* NRRL 3137 and by *Helotium* sp. NRRL 3129 were studied. Both polymers were readily recovered as amorphous gels by precipitation from culture liquors with two parts of ethyl alcohol or methanol. Dried polymeric material was redissolved in water with agitation to give uniform aqueous solutions up to about 1.5% by weight. The polymers were similar in physical properties but possessed different chemical structures. The viscosity of aqueous solutions of each polymer varied from about 50 centipoises at 0.1% to approximately 2,200 centipoises at 1.4% concentrations by weight. Highly viscous solutions at concentrations of 1% or greater behaved like thixotropic gels. Mono-, di-, and trivalent salts, except borate, did not affect viscosity of either polymer. The viscosities were slightly increased by the addition of borate. Autoclaving did not alter the physical properties of neutral polymer solutions. The polymers were stable in acid or alkaline solutions at moderate temperatures but degraded under extremes of *p*H at 70 C or above. Each polymer had a specific rotation of +20° in aqueous dimethylformamide (1:1). The results of acid hydrolysis and periodate oxidation, in conjunction with paper and gas chromatography, indicate that both polymers are branched glucans containing appreciable amounts of β-1,3 linkages.

Davis, Rhodes, and Shulke (1965) have described the laboratory production of two glucans by *Plectania occidentalis* NRRL 3137 and by *Helotium* sp. NRRL 3129. This report describes the physical and chemical properties of the isolated and purified polymers, hereafter referred to as number 3137, produced by *P. occidentalis*, and number 3129, the product of the *Helotium* sp. Polymer 3129 is produced in higher yield and in a shorter fermentation time than is polymer 3137. Accordingly, it appeared to have greater potential as a fermentation product and, therefore, received greater emphasis in structure studies.

MATERIALS AND METHODS

Preparation of polymer solutions. Polymers separated from culture liquors by alcohol precipitation were redissolved in distilled water, dialyzed 3 to 4 days against distilled water, and lyophilized. Solutions of polymer were prepared routinely by shaking solid material with water in a baffled flask on a rotary shaker or in a Waring Blendor. When preparing solutions for viscosity determinations, a small, metal-blade stirrer was used to avoid the harsh conditions encountered in the Blendor treatment. Although stirring required 18 to 24 hr in contrast to 0.5 to 1 min with the Blendor, such a procedure reduced the possibility of mechanical disruption of the polymer molecules. Insoluble matter was removed by straining the solution through a fine mesh screen, and entrained air was removed by centrifugation at high speed for several minutes. The specific concentration was obtained by determining the dry weight of solids in a representative sample.

Physical properties and composition. Viscosity determinations were made with a Brookfield viscometer, type LVF, at spindle speeds of 30 rev/min except where noted. Glucose was measured quantitatively by the method of Shaffer and Somogyi (1933) and by the glucose-oxidase procedure of White and Subers (1961).

Complete methylation of the polymers was not achieved after several attempts. The method described by Gorin and Perlin (1956) was used for the acetolysis of the polymers. Column fractionations of polymer hydrolysates were conducted by the procedure of Goldstein and Whelan (1962) with a 1:1 mixture of Darco G-60 and Celite 503 as packing. Periodate oxidations were patterned after the procedure of Rankin and Jeanes (1954). Enzymatic hydrolytic procedures involving an *exo* and an *endo* β-D-1,3-glucanase were those described by Reese and Mandels (1959). Ultrasonic

treatment of polymer solutions was accomplished with a Raytheon 10-kc sonic oscillator for periods of 1 to 3 hr, with cooling by circulating ice water.

Chromatography. All paper chromatography was done at 25 C by the descending technique with the following solvent systems: *n*-butanol–pyridine–water (6:4:3), isopropyl alcohol-acetic acid-water (67:10:23), and ethyl acetate-acetic acid-pyridine-water (5:1:5:3). Reducing substances were detected by the method of Block, Durrum, and Zweig (1958), although other definitive spray reagents were used as required. Sugars in reaction mixtures were also detected by gas chromatography of their trimethylsilyl derivatives on silicone columns, according to the techniques described by Bentley et al. (1963). Gas chromatograms were prepared with a model 720 F and M instrument equipped with dual 4-ft columns and a thermal conductivity cell detector. Analyses were programmed from 155 to 270 C at 20 C per min at a helium-flow rate of 50 ml/min and pressure of 30 psi.

RESULTS

Physical properties. The relationship of viscosity to polymer concentration for each polysaccharide is shown in Fig. 1. Highly viscous preparations are obtained at polymer concentrations of 1% and above; at such concentrations, polymer solutions have the appearance of soft gels. The viscosity of the polymers is essentially

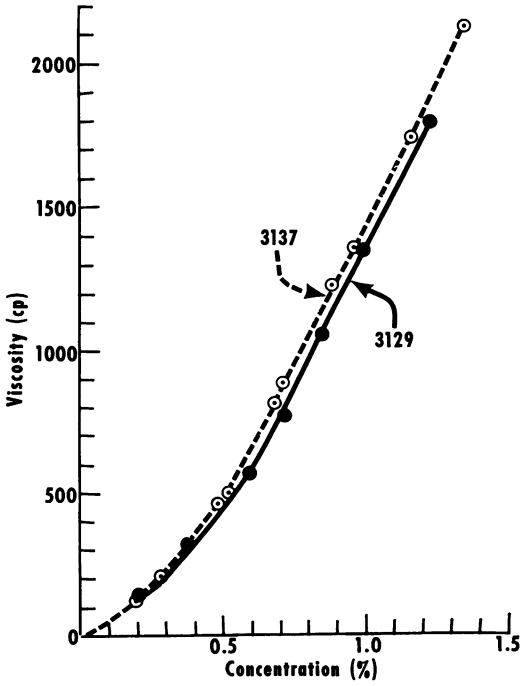

FIG. 1. *Viscosity (in centipoises) of aqueous solutions of polymer 3137 and of polymer 3129 at 25 C.*

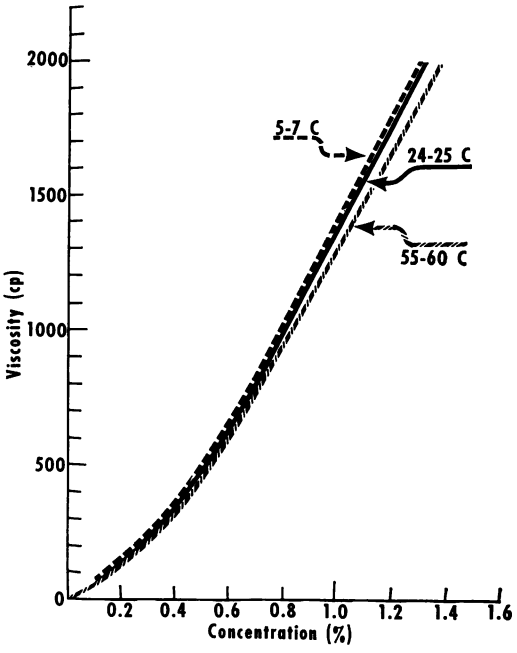

FIG. 2. *Viscosity (in centipoises) of polymer 3129 as a function of temperature and concentration.*

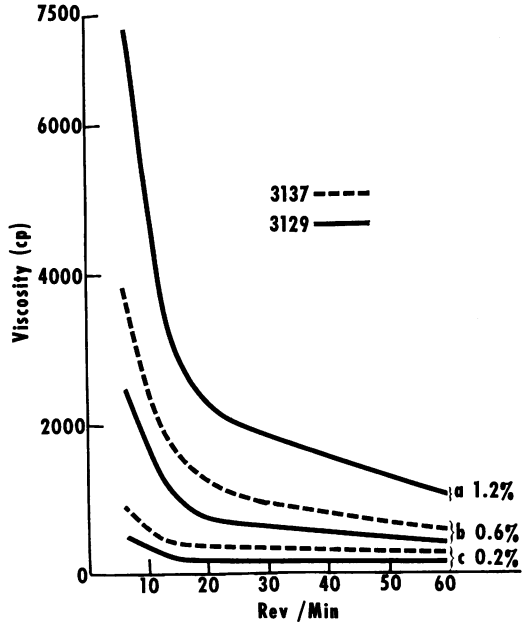

FIG. 3. *Viscosity (in centipoises) of polymer 3137 and of polymer 3129 as a function of concentration and shear.*

unaffected over a wide range of temperature, as illustrated for polymer 3129 in Fig. 2. Errors due to evaporation precluded accurate observations at temperatures higher than those pre-

sented; however, no marked alteration of viscosity was observed at temperatures up to 90 C.

Both polymers exhibited a rapid, initial reduction in viscosity with increasing shear, particularly at concentrations of 1% and above. This characteristic of the polymers is illustrated in Fig. 3.

The addition of 0.1% levels of mono-, di-, and trivalent mineral salts, except borate, to solutions of these polymers caused no significant changes in their viscosity. Representative data for polymer 3137 are shown in Table 1. When added to a polymer solution of lower concentration, tetraborate (0.1%) produced a small, but abrupt, initial increase of viscosity.

The polymers reacted somewhat differently when aqueous solutions were frozen, then thawed after 7 days, subsequently refrozen, and again thawed after an additional 7 days. Solutions containing polymer 3137 showed no alteration of viscosity and appeared normal after such treatment. Solutions of polymer 3129 lost 25 to 30% of their original viscosity if allowed to thaw slowly after 7 or 14 days, apparently because some polymer separated from solution. When polymer 3129 was thawed rapidly, its solutions remained homogeneous and no viscosity loss was observed (Table 2).

A useful attribute of polymers is stability under acid and alkaline conditions. Accordingly, polymer solutions were tested for changes of their viscosity when stored at room temperature and below for 5 to 7 days at *p*H levels from 2.0 to 12.0. Significant changes of viscosity were observed only under strongly alkaline conditions (*p*H 11.0 to 12.0), wherein the viscosity increased approximately 10% within the first 24 hr, then returned to values comparable with solutions tested at lower *p*H levels.

Polymer solutions maintained at temperatures of 70 to 80 C lost viscosity under both acid (*p*H 1.8) and alkaline (*p*H 12.3) conditions, although the rate of loss was greater in acid solutions. In either acid or alkali, the viscosity of polymer 3129 declined more slowly than did that of 3137 (Table 3).

Both polymers withstood extended autoclaving in neutral solution. For example, the viscosity of a 0.3% solution of polymer 3129 changed only from 207 to 262 centipoises after 60 min in an autoclave at 121 C. Addition of calcium chloride plus 30 min of additional autoclaving did not significantly alter the viscosity of the polymer solution.

Although the polymers were stable to acid or alkali at moderate temperatures (Table 3) and to autoclaving under neutral conditions, a 47% loss of viscosity occurred when they were autoclaved in acid or alkali solutions.

Chemical composition. Each polymer lost about 7.5% of its initial air-dried weight when dried at 70 C to constant weight. Carbon and hydrogen analyses of the dried polymers are:

TABLE 1. *Effect of salts on the viscosity of polymer 3137 and polymer 3129*

Salt (0.1%)	Polymer (0.5%)	Viscosity* at 25 C			
		Initial	5 min	24 hr	120 hr
KCl	3137	548	525	540	555
$CaCl_2$	3137	548	518	555	543
$AlCl_3$	3137	548	535	554	545
$Na_2B_4O_7$	3137†	119	124‡	127	
	3129†	105	119‡	121	

* Expressed in centipoises.
† Concentration was 0.2%.
‡ At 60 min.

TABLE 2. *Effect of freeze-thaw cycles on the viscosity of polymers 3137 and 3129*

Thaw rate	Frozen storage time	Viscosity*	
		Polymer 3137	Polymer 3129
	days		
	0	334	344
Slow	7	328	202
	14	319	255
	14†	317	240
Rapid	7	317	331
	14	321	338
	14†	313	332

* Expressed in centipoises.
† Two 7-day freeze-thaw cycles.

TABLE 3. *Effect of acid and alkali at elevated temperature on the viscosity of polymers 3137 and 3129*

Temp	*p*H	Polymer	Viscosity*		
			Initial	8 hr	24 hr
C					
50	1.7	3137	176†	166	167
	11.9	3137	176†	163	257
70–80	1.8	3137	193	159	102
	12.3	3137	799	669	560
	1.8	3129	221	233	176
	12.3	3129	745	717	617

* Expressed in centipoises.
† At 25 C.

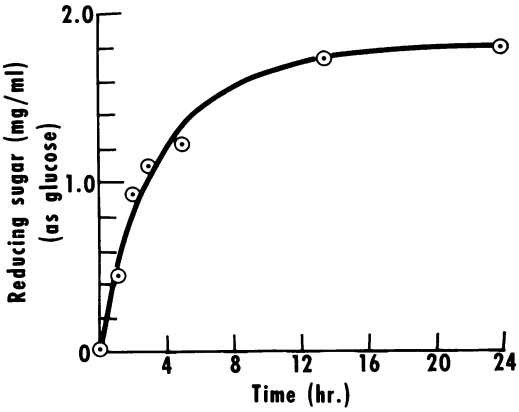

FIG. 4. *Acid hydrolysis of polymer 3137.*

Calculated for $C_6H_{10}O_5$: C, 44.44%; H, 6.17%

Found for no. 3137: C, 44.02%; H, 6.03%; N, 0.57%

Found for no. 3129: C, 42.49%; H, 6.14%; N, 0.43%

Average ash content: for 3137, 2.22%; for 3129, 4.1%

Although the carbon and hydrogen values for 3137 are satisfactory, the value for carbon in polymer 3129 is inexplicably low. The appreciable residual ash content which remains after dialysis indicates a tenacious occlusion or binding of impurities by these polymers, which may be further inferred from the tendency of polymer 3129 to retain color throughout its purification.

Paper chromatography of acid hydrolysates indicated glucose to be the only major component of both polymers. A correlation between polymer weight per milliliter of solution and the concentration of reducing sugar after acid hydrolysis was obtained by refluxing polymer 3137 in 1 N HCl for 22 hr.

Initial polymer concentration: 1.70 mg/ml

Final concentration of hydrolyzed solids: 1.85 mg/ml

Final reducing sugar: 1.66 mg/ml

These values account for approximately 90% of the theoretical sugar, if the polymer is assumed to be composed exclusively of glucose. The rate of acid hydrolysis of polymer 3137 is shown in Fig. 4.

Structure studies. Optical rotations were determined on dilute (0.2%) solutions of polymer in a mixture of dimethylformamide and water (50:50) to alleviate the turbidity inherent in aqueous polymer solutions. At 25 C, the specific rotations of polymers 3137 and 3129 were +20°. In 0.5 N NaOH, polymer 3129 had a specific rotation of −2°, and, in cuprammonium solvent (Reeves, 1944), the rotation of polymer 3129 was +45°.

Structure was initially studied by the periodate-oxidation technique. The production of formic acid by this procedure could arise from either terminal or 1,6-linked glucosidic residues. Polymer 3137 consumed only enough periodate to account for the formic acid produced in the reaction. Approximately 1 mole (33%) of formic acid was produced for every 3 moles of anhydroglucose present. Oxidation of polymer 3129 required periodate in excess of that used for formic acid production. Because the molar ratio of total periodate consumed per mole of anhydroglucose present was less than unity, the polysaccharide must contain unreactive glucose residues.

Fractionation on a carbon-Celite column of the reaction products from the acetolysis of polymer 3137 gave only glucose and small amounts of a sugar which on paper chromatograms had an R_F value similar to either mannose or fructose. No disaccharides or oligosaccharides were observed. Acetolysis of polymer 3129, followed by fractionation on a carbon-Celite column, gave a fraction that produced spots with R_F values on paper chromatograms indicative of uronic acids. No other products were obtained from the reaction mixture.

Susceptibility to hydrolysis by specific enzymes can help establish linkages; furthermore, the structures of indentifiable oligosaccharides often obtained as products of hydrolysis may help define the structure of the parent polymer (Johnson et al., 1963). Active α-1,4- and α-1,6-amyloglucosidase produced by *Aspergillus niger* NRRL 337, incubated at 60 C with an aqueous solution of polymer 3129, liberated approximately 0.4% by weight of glucose. The low yield of glucose indicates the absence of α-1,4- and possibly α-1,6-bonds; nevertheless, the small amount of monosaccharide obtained was expected because the enzyme mixture has a limited ability to attack β linkages as well as α types. Both polymers were hydrolyzed when incubated at 40 C for 24 hr in citrate buffer (*p*H 4.5) with an *exo* β-D-1,3-glucanase produced by Basidiomycete QM 806 (Reese and Mandels, 1959). Chromatography with paper and with gas-liquid techniques of a polymer 3137 hydrolysate, which had been fractionated on a carbon-Celite column, showed that the enzyme had liberated glucose, a trace of fructose, a disaccharide having the same R_F as gentiobiose, and a compound believed to be an oligosaccharide. A similar examination of polymer 3129 showed glucose, fucose, a disaccharide believed to be gentiobiose, and a compound of low R_F value, possibly a uronic acid. Fucose was a minor constituent. In each instance, hydrolysis of both

polymers by this enzyme gave more disaccharide than glucose. Neither polymer was attacked by an *endo* β-D-1,3-glucanase from *Rhizopus arrhizus* QM 1032, although laminarin was degraded readily by this enzyme preparation.

After acid hydrolysis (4 N H_2SO_4, 16-hr reflux), total reducing values calculated as glucose averaged 74% for polymer 3137 and 67% from polymer 3129. When enzyme QM 806 was used (24 hr, 40 C), values for reducing sugar were 37% (polymer 3137) and 17.5% (polymer 3129).

From the differences between values obtained by the glucose-oxidase and Shaffer-Somogyi assays of enzymatic hydrolysates, it was determined that 82% of the total reducing sugar from polymer 3137 was not glucose. For polymer 3129, the corresponding value was 64%. These values confirm the results obtained by paper chromatography, which indicate a preponderance of disaccharide in the enzyme hydrolysate.

By use of the procedures of Abdel-Akher et al. (1952), the polymers were each oxidized with sodium periodate at 7 C for 44 hr, resuspended in water, reduced with sodium borohydride, then hydrolyzed by 0.1 N sulfuric acid at room temperature for 19 hr, and neutralized. Under these conditions, polymer 3137 formed a turbid gel, which was soluble in hot water but retrograded slowly to an insoluble gel on standing. Polymer 3129 gave a turbid solution from which polymer could be separated either by centrifugation for 48 min at 40,000 × *g* or by an ethyl alcohol concentration of 70%, as contrasted to native polymer which becomes insoluble in a 32% alcoholic solution. Additional polymer could be precipitated, after centrifugation, from the resultant clear supernatant solution by further addition of ethyl alcohol. However, dialysis (96 hr) of this supernatant solution resulted in nearly complete loss of alcohol-insoluble material. The treated polymers obtained by the oxidation and reduction procedure were inert to any further action of periodate, indicating that only 1,3-bonds or a combination of both 1,3- and branched glucose units containing no free adjacent hydroxyl groups were present. Subsequent incubation of the treated polymers with QM 806 enzyme gave glucose as the only detectable product on paper chromatograms.

Ultrasonic treatment for 3 hr disrupted the structure of the polymers sufficiently to cause a 99% decrease in the viscosity of aqueous solutions (Fig. 5). A threefold excess of alcohol was added, and the precipitated polymer was washed by decantation. The supernatant solution was fractionated on a carbon-Celite column. Analysis of the fractions by gas chromatography gave peaks with the same retention characteristics as glucose and either fructose or mannose. No evidence was found for disaccharides or larger oligosaccharide fractions which may have resulted from ultrasonic disruption of the polymer. After 3-hr ultrasonic degradation of polymer 3137, an analysis of the product by light-scattering procedures (Senti et al., 1955) indicated an approximate molecular weight of 1.2 million. The specific rotation of the same material in water was +20°, in agreement with the rotation of untreated polymer.

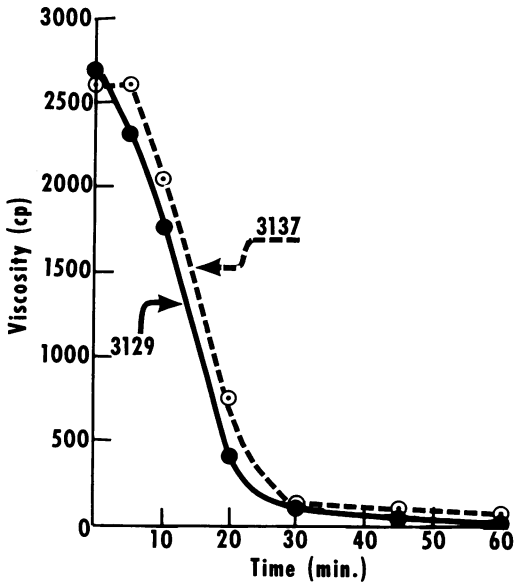

FIG. 5. *Viscosity (centipoises) changes induced by ultrasonic treatment of polymer 3137 and of polymer 3129.*

DISCUSSION

The low specific rotations of polymer 3129 solutions in water and in sodium hydroxide are characteristic of the β configuration and the pyranose ring structure. The low, positive rotation of polymer 3129 in cuprammonium reagent, evaluated with respect to the low rotation in aqueous solvents, is added evidence for the presence of 1,3-glucosidic linkages in the molecule (Reeves, 1944).

Periodate oxidation studies, in conjunction with borohydride reduction and acid hydrolysis, show that 60 to 70% of the glycosidic linkages in polymer 3137 are 1,3, and that the remainder of the molecule contains 30 to 40% of either terminal, nonreducing hexopyranose units or monosaccharides present as 1,6-bonded side chains. Added evidence for the presence of β-1,3-linkages in both polymers is adduced by the ready hy-

drolysis of these polysaccharides by *exo* β-D-1,3-glucanase to give primarily glucose and a disaccharide as major products. The ratio of non-glucose reducing sugar to glucose attained by enzyme hydrolysis could indicate the presence of either a high degree of branching or a main chain composed of glucose units joined not only by 1,3-bonds, but also by 1,6-bonds interspersed among the 1,3-linkages. Were the latter situation widely prevalent, however, the insoluble gel arising from the action of periodate and borohydride would not have been formed as reported. The gel we obtained had solubility properties characteristic of 1,3-linked glucans of high molecular weight, such as laminarin. In the oxidation with periodate, therefore, the disaccharide and any other sugars must have been derived primarily from degradation of the side chains.

In contrast to polymer 3137, periodate oxidation, in association with borohydride reduction and hydrolysis, caused an apparent decrease in molecular weight of polymer 3129 as indicated by increased alcohol solubility and the ability of the product to pass through a dialysis bag (24 A pore size). These results indicate a major degradation of the primary structure of this polymer by periodate oxidation, and show that polymer 3129 must also have bonds other than the 1,3-type present in the main chain. On the basis of periodate uptake and formic acid production data for this polymer, it is tentatively concluded that 25% of the molecule contains 1,6-bonds or a combination of 1,6-bonds and terminal hexopyranose units, 30% of the bonds are 1,2- or 1,4-, or both; and the remainder (45%) are 1,3-bonds.

The viscosity of aqueous polymer solutions may not arise from a long chain length *per se*, but could be the result of an intertwining or crosslinking of branched chains by means of weak intermolecular forces. Within the confines of such an aggregate, one might expect to find occluded monosaccharides such as fructose or mannose which were formed by autolysis of the cell walls of the microorganisms during the fermentation. Thus, ultrasonic treatment of these polymers resulted in loss of viscosity and a concomitant liberation of small amounts of glucose and of extraneous monosaccharides with retention times similar to fructose or mannose. However, the possibility that mannose is present may provide a partial explanation for the small initial increase in the viscosity when tetraborate is added to polymer solutions. The ability of borate to react with mannose at the *cis* C_2 and C_3 hydroxyl groups (Böeseken, 1913) might make it possible for one mannose-containing chain to be thus bonded to a mannose unit of a second chain by borate. The crosslinking of polymer chains in such a manner would probably increase viscosity.

It is proposed that polymer 3137 is a β-1,3-glucan with a branched structure composed largely of anhydroglucopyranose units, but which might contain small amounts of fructose, and possibly mannose. Polymer 3129 has a more complex structure composed of a variety of alternating bond types such that fragmentation of the main chain occurs as a result of oxidation by periodate. A portion of the molecule, however, has contiguous 1,3-bonds, although such continuity of bond type is not sufficient to produce high molecular weight species when subjected to periodate oxidation. Fucose and, probably, a uronic acid are present as either minor constituents or impurities.

Both polymers are of high molecular weight and do not form true solutions. The structure of polymer 3137 resembles other polymers reported by Peat, Whelan, and Lawley (1958), Johnson et al. (1963), Rozenfel'd and Preobrazhenskaya (1962), and Perlin and Taber (1963) in their studies of glucans obtained from seaweed and from microorganisms.

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