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Galactosaminogalactan from Cell Walls of *Aspergillus niger*

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A new heteropolysaccharide has been isolated by alkaline extraction of hyphal walls of *Aspergillus niger* NRRL 326 grown in surface culture. Its composition by weight, as determined by paper and gas chromatography and colorimetric analyses, is 70% galactose, 20% galactosamine, 6% glucose, and 1% acetyl. Two independent experiments have been used to ascertain copolymer structure: permeation chromatography in 6 M guanidinium hydrochloride, with controlled-pore glass columns of two fractionation ranges, and nitrous acid deaminative cleavage of galactosaminogalactan followed by reduction of fragments with [^{3}H]borohydride and gel filtration chromatography. One of the tritiated fragments is tentatively identified as the disaccharide derivative galactopyranosyl 2,5-anhydrotalitol, on the basis of chromatographic properties and by kinetics of its acid hydrolysis. Smith degradation, methylation, deamination, and optical rotation studies indicate that the galactosaminogalactan consists of a linear array of hexopyranosyl units joined almost exclusively by α-(1→4) linkages. Hexosaminyl moieties are distributed randomly along the chains, which have an average degree of polymerization of about 100. The possible significance of this macromolecule in hyphal structure is considered.

Fungal cell walls contain a complex mixture of polysaccharides and peptidoglycans, some of which appear in layers or tiers in the hyphal architecture (1, 27, 32). A few polysaccharides such as glucans and chitin, which are major components of many hyphal walls, function in delineating gross conformation and confer mechanical strength to the wall (16, 27, 28, 32, 51). It is becoming more apparent, however, that most species examined contain, in addition to the above components, a number of other polysaccharides and peptidoglycans in lesser amounts (for reviews, see references 4 and 17). Since hyphae are subjected to changes in nutritional state and morphological characteristics during growth, it is probable that wall polysaccharides reflect or participate in these phenomena (28). However, very little is actually known about either the structures of many of these polysaccharides or their biological functions.

The hyphal wall of *Aspergillus niger* contains glucan (51) and chitin (10) as major components, but also has additional polymeric carbohydrates in small quantities. Previous studies in our laboratory have examined certain aspects of structure and location of nigeran (55, 56) and pseudonigeran (24) in *A. niger*. These α-glucans are found in relatively minor amounts in the hyphal matrix; abundance of nigeran is dependent upon the nitrogen content of the growth medium (19). This communication describes the isolation and partial structural characterization of a novel heteropolysaccharide of *A. niger* hyphal walls that is also present in small amounts. It has a rather unusual constitution, being composed primarily of galactopyranosyl and 2-amino-2-deoxy-galactopyranosyl units.

MATERIALS AND METHODS

Materials. ^{3}H-labeled potassium borohydride was purchased from New England Nuclear Corp., Bio-Gel P-2 (100 to 200 mesh) was purchased from Bio-Rad Laboratories, controlled-pore glass (CPG) beads were obtained from Electro-Nucleonics (CPG 10-350 beads have a mean pore diameter of 31.0 to 39.0 nm and CPG 10-500 have a mean pore diameter of 45.0 to 55.0 nm), guanidinium hydrochloride was obtained from J. T. Baker Co., and precoated thin-layer chromatography cellulose plates were purchased from Brinkmann Instruments. Gas-Chrom P, 100 to 120 mesh, and ECNSS-M liquid phase for gas chromatography were purchased from Applied Science Laboratories. Carbohydrates were obtained from Pfanstiehl Laboratories, and glycogen was purchased from Nutritional Biochemicals Corp. Crystalline threitol and galactocarolose, prepared from *Penicillium charlseii* containing 43% galactofuranosyl residues, were kind gifts from J. E. Gander, University of Minnesota. Methylated galactose standards were generously supplied by G. Aspinall, and methylated galactosamine reference compounds were gifts from P. A. J. Gorin and P. Stoffyn. Lobster chitin was obtained from our labora-

tory collection. Chitosan was prepared from chitin as described by Horton and Lineback (26) (N content from experimental data, 7.6%; calculated for $C_6H_{11}O_4N$, 8.7%). All carbohydrate standards were of the D configuration unless otherwise noted.

Preparation of cell walls. Surface cultures of *A. niger* NRRL 326 were grown from spore inoculum as described previously (56). Mycelial mats were harvested just before sporulation and homogenized briefly in water with a Sorvall Omni-Mixer at 0 C. The dispersed hyphae were centrifuged at 4,000 × *g* to remove growth medium, washed again, suspended in water, and passed twice through a chilled French press (Aminco model 4-3398) to break the cells. Aliquots of the homogenate were stained with methylene blue and examined by light microscopy to ensure that cell breakage was complete. The broken-cell suspension was then washed at 2 C three times each with water, 0.5 M NaCl, and water by centrifugation at 4,000 × *g*. The product was freeze-dried and stored in a desiccator at room temperature.

Isolation of galactosaminogalactan from cell walls of A. niger. Five grams of cell walls was extracted twice with 250 ml of boiling water, the walls were filtered through cheesecloth while hot, and the residue was repeatedly washed with boiling water until free of nigeran, as evidenced by lack of turbidity in the cool filtrate (42). The nigeran-free residue was suspended in 50 ml of water and treated with 50 mg of $NaBH_4$. Reduction of the insoluble residue with $NaBH_4$ is employed to protect polysaccharide chains from degradation during subsequent steps in which alkali is employed. A second 50-mg portion of $NaBH_4$ was allowed to stand overnight. Excess borohydride was destroyed by dropwise addition of cold acetic acid to the chilled suspension (final pH, 5). The walls were then centrifuged at 16,000 × *g* for 10 min at 4 C and washed repeatedly with water. They were then suspended in 100 ml of water, and 100 ml of 2 NaOH was added. The mixture was autoclaved for 3 h without pressure, cooled, and centrifuged at 16,000 × *g* for 10 min at 4 C. The supernatant solution was stored at 0 C, and the residue was reextracted at room temperature with 200 ml of 1 N NaOH with stirring. To the combined alkaline supernatant solutions, methanol (3 volumes) was added with stirring, and the resulting precipitate was allowed to flocculate overnight at room temperature. The precipitate was collected by centrifugation, washed with ice-cold 1 N acetic acid, exhaustively dialyzed against distilled water at 2 C, and lyophilized (yield, 0.382 g; 7.64% based on dry weight of cell walls).

The crude lyophilized polysaccharide preparation (0.382 g) was suspended in 100 ml of water and stirred for 2 to 3 h at room temperature. The mixture was centrifuged 20 min at 16,000 × *g* at 4 C. This washing was repeated until the wash water was free of phenol-sulfuric acid-positive materials. The combined supernatants were then exhaustively dialyzed against water and lyophilized, giving a water-soluble fraction rich in galactose, glucose, and mannose (yield, 0.088 g; 1.76% based on dry weight of cell walls). The precipitate was also lyophilized. This material was extracted with 1 N NaOH (200 ml) with stirring at room temperature for 5 h. The supernatant was removed, and the residue was reextracted with 1 N NaOH as before. The combined alkaline solutions were exhaustively dialyzed and then lyophilized to obtain a glucan fraction (yield, 0.264 g; 5.28% based on dry weight of cell walls).

The residue remaining after removal of most extractable glucan was again extracted with 200 ml of 1 N NaOH by stirring at room temperature for a 5-h period. This process was repeated until the supernatant was devoid of phenol-sulfuric acid-positive components. The final residue was then exhaustively dialyzed against distilled water and lyophilized to concentrate the galactosaminogalactan (yield, 0.030 g; 0.6% based on dry weight of cell walls). The isolation procedure is summarized in Fig. 1.

Analytical procedures. Neutral hexose was determined by the phenol-sulfuric acid method (14), reducing power was determined by the Nelson-Somogyi procedure (49), and amino sugars were visualized by ninhydrin (36). Quantitation was accomplished with a galactose standard in the first two assays and with galactosamine hydrochloride in the last. Nitrogen analyses were performed by the Microanalysis Laboratory of the University of Massachusetts. *N*-acetyl determinations were made by the procedure of Radhakrishnamurthy et al. (41), and *N*-acetylation of the galactosaminogalactan was accomplished by the method of Perlin (39), which included peracetylation of the polymer followed by de-*O*-acetylation with 0.2 N KOH at room temperature. The final product was water insoluble.

Optical rotations were measured with a Zeiss polarimeter, radioactivity was determined with a Packard model 3310 liquid scintillation spectrometer with techniques described previously (24), infrared spectra were recorded with a Perkin Elmer model 257 spectrophotometer, and a Varian aerograph model 2800 with a flame ionization detector was employed for gas-liquid chromatography.

Paper and thin-layer chromatography. Paper chromatography (descending) was conducted with Whatman no. 1 filter paper and the following solvent systems: system 1, pyridine-ethyl acetate-water (2:5:7, vol/vol, upper phase); system 2, 2-butanone–acetic acid-boric acid saturated water (9:1:1, vol/vol); system 3, nitromethane-absolute ethanol-water-acetic acid (35:41:23:10, vol/vol); system 4, ethyl acetate-acetic acid-formic acid-water (19:3:1:4, vol/vol) (28); system 5, pyridine-ethyl acetate-water (11:40:6, vol/vol) (49); system 6, *n*-butyl alcohol-acetic acid-water (4:1:5, vol/vol); and system 7, *n*-butyl alcohol-ethanol-pyridine-water (40:11:2:19, vol/vol) (22). Reducing sugars were visualized with the alkaline $AgNO_3$ reagent (54), sugar alcohols were visualized with benzidine-periodate spray, and amino compounds were visualized with ninhydrin. Hexosamines were also detected as bright fluorescent spots under ultraviolet light after the paper had been dried in an oven at 150 C for 15 min (12). After this treatment, hexosamines did not react with ninhydrin reagent but were made visible as a black spot with alkaline $AgNO_3$ reagent. Thin-layer chromatography on cellulose-coated plates was ac-

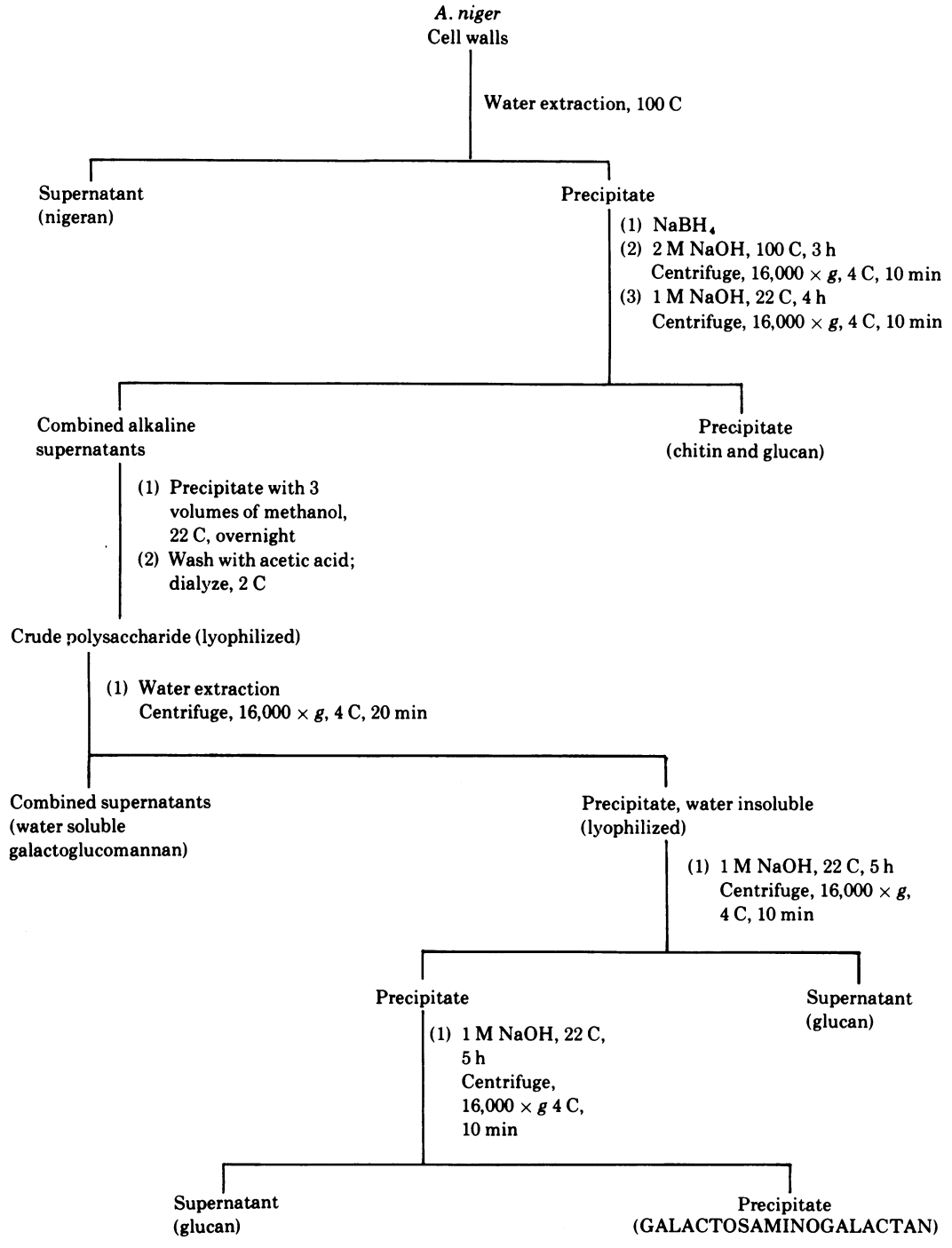

FIG. 1. *Purification procedure for isolation of galactosaminogalactan from A. niger NRRL 326 cell walls.*

complished with solvent system 2. Components were visualized by exposing the plate to iodine vapor or with reagents employed in paper chromatography.

Column chromatography. For permeation chromatography of intact galactosaminogalactan, a 5-mg sample was dissolved in 1 to 2 ml of 6 M guanidinium hydrochloride solution with stirring, applied to columns (1 by 95 cm) of CPG 10-500 or CPG 10-350, and

eluted with 6 M guanidinium hydrochloride. Aliquots of column fractions were monitored for neutral sugars by the phenol-sulfuric acid assay. After dialysis against water, selected pooled fractions were acid-hydrolyzed and analyzed for both neutral and amino sugars. Nitrous acid-degraded galactosaminogalactan was chromatographed on Bio-Gel P-2 columns (either 1 by 200 cm or 1.4 by 50 cm). Solutions, 0.5 to 2 ml containing 5 mg of samples, were applied to the columns and eluted with water. Elution profiles were monitored for either neutral sugar or radioactivity.

Gas-liquid chromatography. Sugars and products of Smith degradations were analyzed as their alditol acetates (37, 46, 51) with columns packed with 3% ECNSS-M on Gas-Chrom P. Neutral sugars were analyzed on a stainless-steel column (3 mm by 180 cm); methylated and amino sugars were analyzed on a glass column (3 mm by 120 cm). Calibrations for relative detector responses of authentic compounds were made for each column. All samples were introduced onto the columns in chloroform solution.

Acid hydrolysis. Hydrolyses of the polysaccharide or its derivatives were performed in Teflon-lined screw-cap vials at 100 C. Complete hydrolysis was accomplished with 1 N HCl under N_2 (35) or by methanolysis and acid hydrolysis as described below for the methylated polymer. Release of hexosamines as determined by ninhydrin reached a maximum value between 70 and 90 min of heating in 1 N HCl. When possible, acid was removed by lyophilization of hydrolysates over NaOH pellets. Mild acid hydrolysis was conducted with 0.01 N or 0.2 N H_2SO_4 or 0.2 N HCl at 100 C. Kinetics of acid hydrolysis were determined by removing aliquots of the polymer suspension at various times and neutralizing with 1 N NaOH. The chilled hydrolysates were centrifuged at 1,500 $\times$ *g* for 5 min to remove insoluble polysacchride before those analyses where the supernatant contents were examined.

Methylation. Methylation was performed on a 50-mg sample of the fully *N*-acetylated galactosaminogalactan as described by Lindberg (30). After treatment of the polymer with methyl iodide, water was cautiously added to the reaction mixture to destroy excess sodium hydride. It was then dialyzed exhaustively against water and lyophilized. Two reaction sequences were employed to completely methylate the polysaccharide, as judged by the absence of the characteristic hydroxyl band at 3,500 to 3,600 cm^{-1} in the product's infrared spectrum. The final yield was 32 mg ($[\alpha]_D^{125}$, c 0.13 $CHCl_3$). Permethylated polysaccharide was depolymerized by methanolysis in 5% methanolic hydrochloride under reflux overnight followed by acid hydrolysis in 3 N HCl at 100 C for 2 h (20). The resulting methylated monomeric sugars were analyzed after borohydride reduction and acetylation (7, 8) by gas chromatographic analysis with the glass column.

Periodate oxidation. Galactosaminogalactan was oxidized in the dark at room temperature with unbuffered 0.015 M sodium metaperiodate solution (initial pH, 5). At least 1.5 mol of periodate was initially present for 1 mol of anhydrohexose unit of polysaccharide oxidized. The reduction of periodate was measured spectrophotometrically (2). A control oxidation of nigeran was employed to check the stoichiometry of periodate uptake.

Smith degradation. Periodate oxidations were conducted on 5-mg samples as described above until total uptake remained constant. After addition of ethylene glycol to destroy excess periodate, the polyaldehyde was dialyzed. Reduction of the polyaldehyde was effected by KBH_4, and when labeled products were desired the pH of the mixture was carefully adjusted to 7.5 to 8 with dilute ammonia, and $[^3H]KBH_4$ was added. After 30 min at room temperature the reduction was completed by the addition of excess KBH_4. The reaction mixture was allowed to stand overnight at room temperature, and excess borohydride was then destroyed by addition of 4 M acetic acid to pH 5. The polyalcohol was then repeatedly evaporated to dryness in the presence of methanol to remove methyl borate and dialyzed against distilled water at 4 C. Complete hydrolysis of the polyalcohol was carried out in 1 N HCl at 100 C overnight, with subsequent removal of acid by lyophilization over NaOH pellets. Mild acid hydrolysis was conducted with 0.25 N H_2SO_4 or 0.25 N HCl at room temperature, and the kinetics of hydrolysis were ascertained by monitoring formation of glycerol and threitol by paper chromatography in solvent system 1 or by thin-layer chromatography with solvent system 2 (15). About 16 h were required for maximal release of threitol from polyalcohol.

Nitrous acid deaminative degradation. Deaminative cleavage of galactosaminogalactan and chitosan were performed by a modification of the procedure of Lindahl et al. (31). A 5-mg sample of polysaccharide was treated at room temperature with 2.5 ml of 3.9 M $NaNO_2$ in 0.3 M acetic acid. Whereas chitosan was completely solubilized in 10 min, the galactosaminogalactan required about 5 h to dissolve. A small amount of insoluble material from the galactosaminogalactan was discarded, and the supernatants of both reactions were neutralized with sodium carbonate and reduced with $[^3H]KBH_4$ and KBH_4 at room temperature overnight. Excess borohydride was destroyed with acetic acid and removed as described above. Gel filtration of products was performed without deionizing, and radioactivity profiles were monitored. Where necessary, fractions containing salt, as determined by conductivity, were pooled and deionized by column chromatography on IR120(H^+) and IR45(OH^-) before further analysis.

RESULTS

Constitution of the polysaccharide. The galactosaminogalactan represents about 0.6 to 0.8% of hyphal wall dry weight, based on four preparations isolated. The final product is insoluble in water, hot 1 N alkali, dimethyl sulfoxide, dimethylformamide, and 8 M urea, but will dissolve slowly in 6 M guanidinium hydrochloride. During the fractionation summarized in

Fig. 1, the polymer appears to be tightly complexed with a glucan fraction, but the mixture was resolved by exhaustive extraction of glucan with alkali. Despite the solubility of purified polysaccharide in guanidinium hydrochloride, it was not possible to extract galactose directly from walls with this solvent even after heating to 100 C, also suggesting that the polysaccharide is tightly bound in the cell wall matrix.

A summary of analytical data is presented in Table 1. More than 96% of the dry weight of the polysaccharide is accounted for by the analyses listed. Calculations from acetyl analysis indicates only 20% of the galactosamine units are *N*-acetylated. However, due to the alkaline conditions of the isolation procedure the actual *N*-acetyl content of the polymer in its native state may differ from this value (53).

Paper chromatography of neutralized acid hydrolysates of the galactosaminogalactan (obtained without ion-exchange columns) with various solvent systems was employed for qualitative determination of monosaccharide constitution. Chromatography in solvent system 1 followed by visualization with silver nitrate revealed two major components, one with a mobility identical to galactose and a second migrating slightly slower than standard glucosamine. The slow component also gave a position reaction with ninhydrin. A small glucose spot was also present (see Fig. 8 and 9). Since this solvent does not separate the hexosamines to any degree, the hydrolysate was analyzed in solvent system 5, in which galactosamine migrates more slowly than glucosamine and mannosamine standards, which do not separate. Results of this experiment indicated that the amino sugar component has a mobility relative to glucosamine of 0.78, identical to that of a galactosamine standard. Additional chromatographic studies with solvent systems 1, 2, and 6 ruled out the possibility that this ninhydrin-positive component observed is aspartic acid, serine, or threonine. Identity of the sugar was confirmed by gas-liquid chromatography of alditol acetates also prepared from HCl hydrolysates of the polysaccharide (Fig. 2 and 3). By employing molar response factors, the ratio of galactose to glucose in the polysaccharide was also calculated (Table 1) with these data.

TABLE 1. *Composition of galactosaminogalactan*

Analysis	Method	% Dry wt
Nitrogen	Microanalysis	1.67[a]
Neutral sugar	Phenol sulfuric acid	75[b]
Amino sugar	Ninhydrin	20[c]
Acetyl	Gas chromatography	1.03
Optical rotation	Polarimetry	$[\alpha]_D^{20}$ + 120°[d]

[a] Theoretical hexosamine content of 20% = 1.70% N.

[b] A galactose standard was employed. The neutral sugar portion is composed of 92% galactose and 8% glucose (determined by gas-liquid chromatography with the conditions given in Fig. 3).

[c] Determined after acid hydrolysis of polysaccharide in 1 N HCl under N_2 at 100 C for 80 min. A galactosamine standard was used in the ninhydrin assay. The amino sugar component is exclusively galactosamine (determined by gas-liquid chromatography with the conditions given in Fig. 2).

[d] As permethylated derivative (c 0.128, $CHCl_3$).

Ring conformation of constituent sugars. The kinetics of hydrolysis in acid were used to characterize the ring conformation of the constituent sugars of the polymer. Figure 4 compares results of mild acid hydrolysis of galactosaminogalactan and galactocarolose, a fungal galactomannan containing its galactose (43%) exclusively as β-(1→5)-linked furanosyl residues: the remaining carbohydrates are mannopyranosyl units (18). The unstable conformation of galactofuranose moieties results in the release of 90% of galactose from galactocarolose in 20 min, whereas only about 10% of the potential reducing groups of galactosaminogalactan are liberated after 100 min of hydrolysis. Stronger acid concentration caused a more rapid release of both neutral carbohydrate and reducing power from the polysaccharide, however (Fig. 5). Whereas 90% of the total neutral hexose in the polymer is *solubilized* in 30 min under these

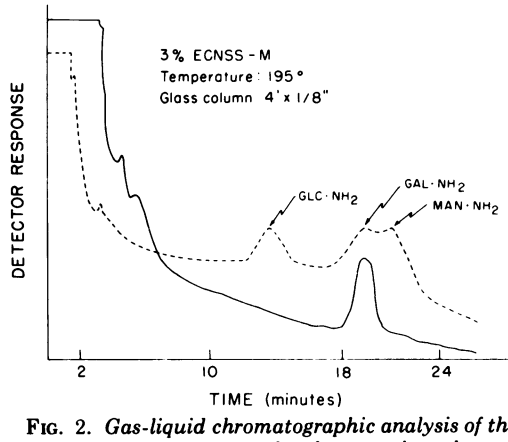

FIG. 2. *Gas-liquid chromatographic analysis of the amino sugar component of galactosaminogalactan. Three milligrams of polymer was hydrolyzed in 1 ml of 1 N HCl under nitrogen at 100 C for 15 h. The mixture was derivatized as alditol acetates as described in the text. The elution positions of authentic amino sugar alditol acetates are shown in the dashed-line profile for comparison.*

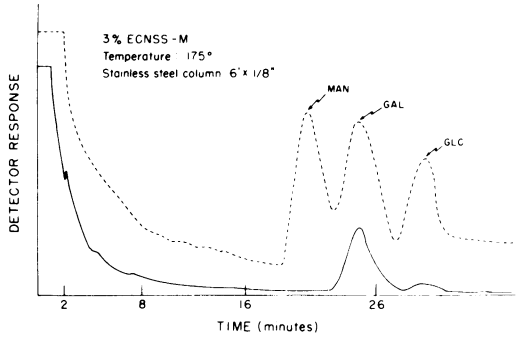

FIG. 3. *Gas-liquid chromatographic analysis of the neutral sugar components of galactosaminogalactan. The elution positions of authentic neutral sugar alditol acetates are shown in the dashed line profile for comparison. For details see text and Fig. 2 legend.*

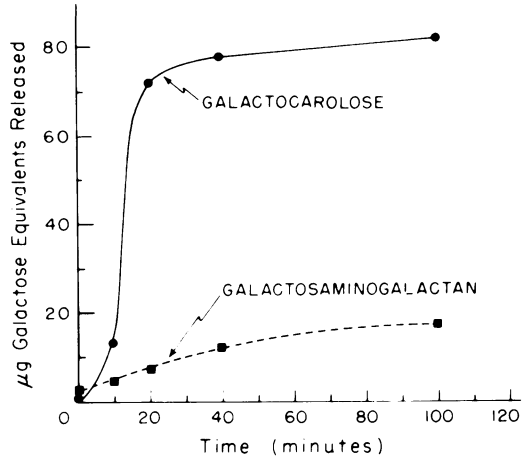

FIG. 4. *Dilute acid hydrolysis of galactosaminogalactan and galactocarolose. Two milligrams each of the two polysaccharides was added to 2.2 ml of water. After withdrawing duplicate 0.2-ml zero time samples, 0.2 ml of 0.1 N H_2SO_4 was added to each hydrolysis mixture, and they were heated at 100 C in Teflon-capped vials. Duplicate 0.2-ml aliquots of the mixtures were withdrawn periodically and assayed for reducing power. The theoretical galactofuranosyl content of a 0.2-ml aliquot of this preparation of galactocarolose is 77 μg (based on 43% galactose). A 0.2-ml aliquot of galactosaminogalactan contains 126 μg of galactose (based on 70% galactose). A galactose standard was used in this experiment.*

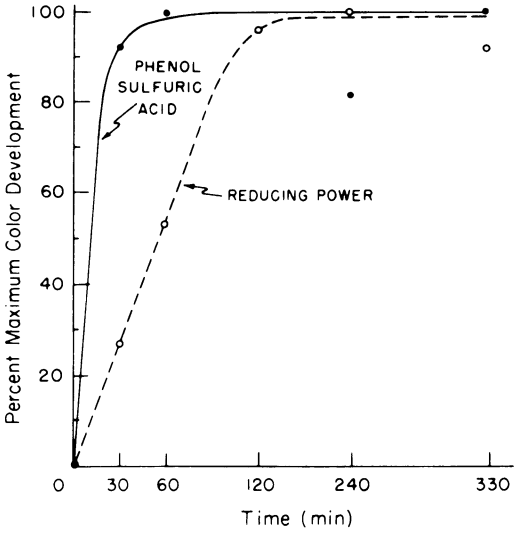

FIG. 5. *Acid hydrolysis of galactosaminogalactan. Polysaccharide, 4.2 mg, was hydrolyzed in 5 ml of 0.2 N H_2SO_4 at 100 C in a Teflon-capped vial. At indicated times, 0.5-ml aliquots were withdrawn and immediately neutralized with 1 N NaOH. They were then chilled and centrifuged at 1,500 × g for 5 min, and the supernatants were assayed for total soluble carbohydrate by the phenol-sulfuric acid assay and reducing power. For details, see text.*

conditions, reducing equivalents are released over a somewhat longer period. The relationship between total carbohydrate solubilized and reducing equivalents suggests that oligosaccharides are being produced initially, and these undergo further hydrolysis during the heating period. The behavior of the polysaccharide under these hydrolysis conditions was used to investigate the possibility that the extreme insolubility of the galactosaminogalactan might retard its hydrolysis in dilute acid. A sample of polysaccharide was heated 30 min at 100 C in 0.2 N H_2SO_4, liberating soluble oligosaccharides. It was then diluted with boiling water to give a final acid concentration of 0.01 N, and heating was continued. Although 40% of the theoretical reducing equivalents had been solubilized at the time of dilution, no further significant hydrolysis occurred during the subsequent 30-min heating period. It is clear that the acid hydrolysis kinetics observed with galactosaminogalactan (including rate of release of galactosamine) support the assignment of a pyranosyl conformation for its saccharide units (5, 34, 37).

Permeation chromatography. Due to its insolubility, the possibility that the galactosaminogalactan might be a mixture of two co-fractionating polysaccharides was considered. Solubilization of galactosaminogalactan in 6 M guanidinium hydrochloride permitted an examination of the size distribution of molecules in the preparation and an evaluation of the ratio of the two major sugars in the various parts of the elution profile. Permeation chromatography was performed with CPG beads with two different fractionation ranges (10–350 and 10–500) equilibrated with 6 M guanidinium hydrochloride. Results with CPG 10–500 are

shown in Fig. 6. The elution profile of galactosaminogalactan is superimposed on a calibration profile of standard galactose and glycogen. The neutral hexose profile indicates a single component eluting between the two markers. Certain fractions across the peak were exhaustively dialyzed to remove guanidine and then analyzed directly by the phenol-sulfuric acid assay and, after acid hydrolysis, by ninhydrin. A similar profile with an essentially constant ratio of ninhydrin to phenol-sulfuric acid absorbances was obtained with CPG 10-350 (data not shown). This result suggests that the preparation is either a copolymer containing galactose and galactosamine or two homopolysaccharides with identical solubilities, fractionation properties, and size distribution characteristics on these two columns.

Deaminative cleavage studies. Nitrous acid deaminative cleavage of the polysaccharide was employed to confirm the presence of galactosamine and galactose in the same molecule. The glycosidic linkage joining an amino sugar, that is not *N*-acetylated, to any other monosaccharide unit, is quantitatively cleaved (31, 47), with concomitant conversion of the amino sugar to a 2,5-anhydroaldose. This derivative can be reduced with borohydride to yield its corresponding 2,5-anhydroalditol. If galactosamine is linked covalently to galactose then derivatives containing one or more galactose units, each having 2,5-anhydrotalitol as its reducing terminal unit, would result. Figure 7 shows the radioactive profiles, from a Bio-Gel P-2 column (50 by 1.4 cm), of products obtained by deaminative cleavage of galactosaminogalactan and a chitosan control. The galactosaminogalactan afforded a number of tritiated components of widely differing molecular sizes, clearly indicating covalent bonding between galactosamine and resistant saccharide units of varying lengths. Conversely, degradation of chitosan, a homopolymer of 2-amino-2-deoxy-D-glucopyranosyl units, gave a single low-molecular-weight fraction (47) eluting at a position near standard galactose. After pooling of appropriate fractions, deionization, and concentration, this low-molecular-weight product was identified as 2,5-anhydromannitol by paper chromatography in solvent system 1, by comparison with an authentic specimen prepared by diazotization of glucosamine hydrochloride (47). Total acid hydrolysis of pooled fractions 20 to 27 from the galactosaminogalactan degradation experiment afforded a single radioactive component that chromatographed in solvent systems 1 and 4 in a manner indistinguishable from 2,5-anhydrotalitol prepared by diazotization of authentic galactosamine hydrochloride. An unhydrolyzed control sample of this pooled fraction gave a single radioactive spot at the origin of both chromatograms. Greater resolution of the products of deaminative cleavage of galactosaminogalactan was achieved on a Bio-Gel P-2 column (203 by 0.9 cm) (Fig. 7b). Elution positions of some carbohydrate standards are superimposed on this data. Comparison of the ^3H elution profiles from two separate diazotization experiments showed that they were quite reproducible. This suggests that the fragmentation of the polysaccharide is probably complete under these conditions.

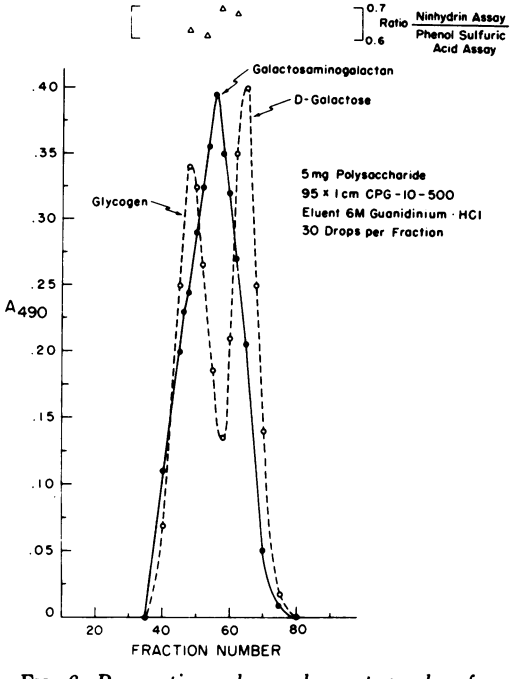

FIG. 6. *Permeation column chromatography of galactosaminogalactan with CPG beads (CPG 10-500) of mean pore diameter 45.0 to 55.0 nm. Selected fractions were dialyzed against water to remove guanidinium hydrochloride. The ratios of color development in the ninhydrin and phenol-sulfuric acid assays for these fractions are indicated by the triangles above the profile. The elution profiles of glycogen and galactose standards are given by the dashed line. A flow rate of 0.2 ml/min was used. A_{490}, Absorbance at 490 nm of fractions in the phenol-sulfuric acid assay.*

Analysis of deaminative cleavage fragments. Peak V (Fig. 7b), which eluted just before standard galactitol, was pooled, deionized, and freeze-dried. Chromatography of an aliquot in solvent system 1 indicated about 80% of the radioactivity corresponds to 2,5-anhydrotalitol. The remainder was divided about equally among three slower moving fractions,

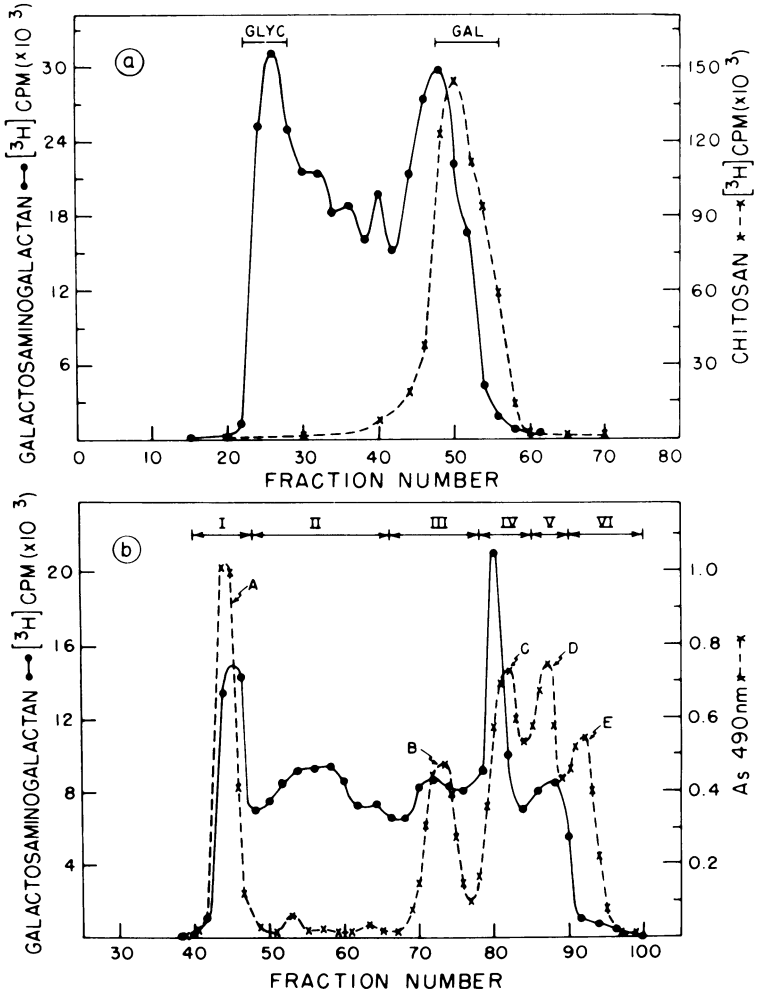

FIG. 7. *(a) Gel filtration chromatography of deamination products of galactosaminogalactan (●) and chitosan (x) after reduction with [³H]borohydride. A 1-ml sample was applied to a column (1.4 by 44 cm) of Bio-Gel P-2 (100 to 200 mesh) and eluted with water. A flow rate of 0.2 ml/min was employed, and 30-drop fractions were collected. The column was calibrated with glycogen and galactose, whose elution positions were determined by the phenol-sulfuric acid assay. (b) Gel filtration chromatography of deamination products of galactosaminogalactan (●) after reduction with [³H]borohydride. A 1-ml sample was applied to a column (0.9 by 203 cm) of Bio-Gel P-2 (120 mesh) and eluted with water. A flow rate of 0.2 ml/min was employed, and 30-drop fractions were collected. An elution profile of a mixture of carbohydrate standards (x) was obtained by the phenol-sulfuric acid assay. (A) Glycogen (Glyc), (B) glucopyranosyl (1→3)glucopyranosyl (1→4) glucopyranosyl (1→3) glucose, (C) melezitose, (D) maltose, (E) galactose (Gal).*

found to have mobilities identical to three of the constituents of peak IV as revealed by paper chromatography in solvent system 1. Neither peak V nor VI contained any [³H]galactitol; therefore no significant hydrolysis of galactose residues occurs under the conditions for deaminative cleavage employed in this study. Similarly, the absence of [³H]galactitol from the acid hydrolysate of fractions 21 to 27 shown in Fig. 7a rules out the formation of large fragments bearing galactose as reducing groups. The small proportion of free [2,5-³H]anhydrotalitol (Fig. 7b) in the total product distribution indicates that a few de-*N*-acetylated galactosamine residues in the polysaccharide are positioned consecutively and/or occupy a nonreducing terminal location in the chains.

Peak IV (Fig. 7b) was found to consist of four

tritiated components when examined by paper chromatography in solvent system 1. Composition and chromatographic data for this peak are given in Table 2. Fraction C was isolated from this mixture by preparative paper chromatography in solvent system 1 and shown to be a single ^3H component in this system in solvent system 4 (Table 2). Due to the small amount of the material isolated, it has not yet been possible to characterize it chemically. Preparation of larger quantities of all the various fragments are now in progress. However, certain experimental evidence favors a tentative identification of fraction C as the disaccharide derivative, galactopyranosyl 2,5-anhydrotalitol. Data in Table 2 show that its chromatographic behavior is indicative of a disaccharide structure. It has a mobility that is faster than maltose but slower than galactitol in both systems 1 and 4 (47). When this fraction is subjected to acid hydrolysis and paper chromatography under conditions detailed in Table 3, tritium is recovered from areas corresponding to either starting material (fraction C) or 2,5-anhydrotalitol at all times during the hydrolysis. This result argues against fraction C having a degree of polymerization greater than 2, since [2,5-^3H]anhydrotalitol is the sole ^3H product even after 10 min of hydrolysis. Similar experiments conducted with fractions of larger molecular size obtained from both Bio-Gel columns give a mixture of tritiated products under these conditions. The kinetics of acid hydrolysis of fraction C (50% hydrolysis in 8 min, Table 3) are similar to that seen with the intact polymer. Since galactose is the only monosaccharide detected by both paper and gas chromatography during early stages of acid hydrolysis of the polysaccharide (0.1 N acid), this hydrolysis characteristic is also consistent with the presence of a galactose component in fraction C. Finally, a sample of fraction C hydrolyzed in 1 N H_2SO_4 for 60 min was reduced with [^3H]KBH_4, acidified and deionized in the usual way. Paper chromatography of the hydrolysate was performed in solvent system 1, and the major radioactive peak detected had a mobility identical to galactitol. There were no peaks corresponding to either fraction C or galactosaminitol and *N*-acetylgalactosaminitol standards. This result suggests that a new molecule reducible with borohydride is generated by acid hydrolysis and that the reduced product is galactitol.

TABLE 2. *Composition of peak IV from a Bio-Gel P-2 column*[a]

Determinant	% of total ^3H in peak	$R_{galactitol}$	
		Solvent 1	Solvent 4
Fraction			
A	29	0.07	
B	20	0.30	
C	38	0.77	0.67[b]
D	13	1.85	
Standards			
2,5-Anhydro-D-talitol		1.85	1.65
2,5-Anhydro-D-mannitol		2.12	
Maltose		0.62	0.32
Galactitol		1.00	1.00
Lactose			0.23

[a] As shown in Fig. 7, peak IV was separated into components A to D by (23-cm) preparative paper chromatography. The sample was applied as 9-inch (23-cm) streaks to three sheets of chromatography paper. Marker lanes containing aliquots of peak IV (as well as suitable standards) were used to locate the radioactivity. Appropriate bands from the papers corresponding to the four areas were eluted with water, pooled, and lyophilized. Radioactive material corresponding to fraction C was then analyzed in solvent systems 1 and 4 and utilized in the experiment described in Table 3.

[b] Purified fraction C migrated as a single radioactive compound with the indicated mobility in solvent system 4.

Periodate oxidation and Smith degradation of galactosaminogalactan. Periodate oxidation of the galactosaminogalactan resulted in the uptake of 0.72 to 0.75 mol of periodate per mol of anhydrohexose, whereas a control oxidation with nigeran afforded 0.45 to 0.47 mol of periodate per mol of anhydrohexose (theoretical

TABLE 3. *Acid hydrolysis of fraction C*[a]

Time (min)	% ^3H migrating at:	
	$R_{galactitol}$ = 0.78	$R_{galactitol}$ = 1.85
0	100	0
10	44	56
20	21	79
40	9	91

[a] A 2-ml sample of purified fraction C from peak IV was hydrolyzed in 1 N HCl at 100 C. At various time intervals, aliquots were withdrawn, neutralized, and chromatographed in solvent system 1. The *entire* length of the chromatogram was cut into strips and counted by liquid scintillation spectrometry. Standards of galactitol and 2,5-anhydro-D-talitol were run as markers with each sample. The total migration of galactitol was 12.5 cm; 2,5-anhydro-D-talitol, 27 cm. The percentage of radioactivity corresponding to each of these standards was then determined.

value for nigeran, 0.5 mol). The result with galactosaminogalactan suggests that approximately one of four or five sugar residues in the polymer is resistant to periodate oxidation (see below).

A Smith degradation was performed on a completely *N*-acetylated derivative of galactosaminogalactan. Comparison of products from acid hydrolysis of intact and Smith-degraded polymers by paper chromatography (Fig. 8) revealed that galactose is largely destroyed by periodate oxidation and that a new component, with a mobility essentially identical to a standard of erythritol, was formed. This component was later identified as threitol by gas-liquid chromatography, however (Table 4).

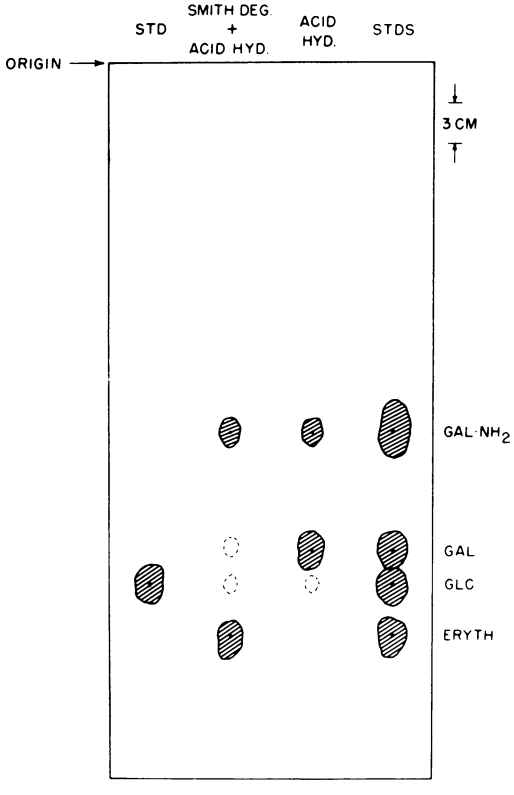

FIG. 8. *Paper chromatography (descending) of acid-hydrolyzed, completely N-acetylated galactosaminogalactan (Acid Hyd.) and products generated after complete acid hydrolysis of periodate-oxidized and borohydride-reduced polymer (Smith Deg. + Acid Hyd.). Visualization was accomplished by silver nitrate dip. Major products and standards are indicated by cross-hatched regions; areas surrounded by a dashed line are minor products. Standards: GalNH$_2$, galactosamine; Gal, galactose; Glc, glucose; Eryth, erythritol. Chromatography was conducted in solvent system 1 for 15 h.*

Glucose and galactosamine appeared relatively resistant to attack under the conditions employed. Quantitative analysis by gas-liquid chromatography (Table 5) confirmed these observations, showing that no galactosamine or glucose but 93% of the galactose was in fact destroyed. Partially *N*-acetylated polysaccharide was also subjected to periodate oxidation and borohydride reduction as above. Paper chromatography of complete acid hydrolysis products of the Smith degradation is shown in Fig. 9. The mixture was found to consist of threitol with very minor amounts of galactose, glucose, and galactosamine. The destruction of galactosamine in this case was confirmed by gas-liquid chromatography also (see below). Results of the two Smith degradations indicate, therefore, that both galactose and galactosamine moieties of the isolated biopolymer are largely susceptible to periodate oxidation and on subsequent reduction and acid hydrolysis give rise to threitol as a major product. However, galactosamine units of the completely *N*-acetylated polysaccharide survive periodate oxidation (Fig. 8, Table 5).

TABLE 4. *Gas chromatographic analysis of the unknown component of Smith degradation*

Component[a]	Retention time[b] (min)
Unknown	2.8
Erythritol	2.2
D-Threitol	2.8
Unknown + erythritol	2.2, 2.8
Unknown + D-threitol	2.8
Glycerol	With solvent front
D-Arabinitol	8.4

[a] As its alditol acetate.

[b] Glass column (4 feet by 1/8 inch [110 by 0.31 cm]), 3% ECNSS-M on Gas-Chrom P, 100 to 120 mesh; column temperature, 145 C.

TABLE 5. *Gas-liquid chromatographic analyses of alditol acetates prepared after acid hydrolysis of completely N-acetylated galactosaminogalactan before and after Smith degradation*[a]

Peak area normalized for internal standard	Intact	Smith-degraded
Galactose	100	7.5
Glucose	7	7
Galactosamine	100	125

[a] Conditions used are indicated in Fig. 2 and 3. An internal standard of i-inositol was carried through the acetylation and reduction steps for quantitation of peak areas.

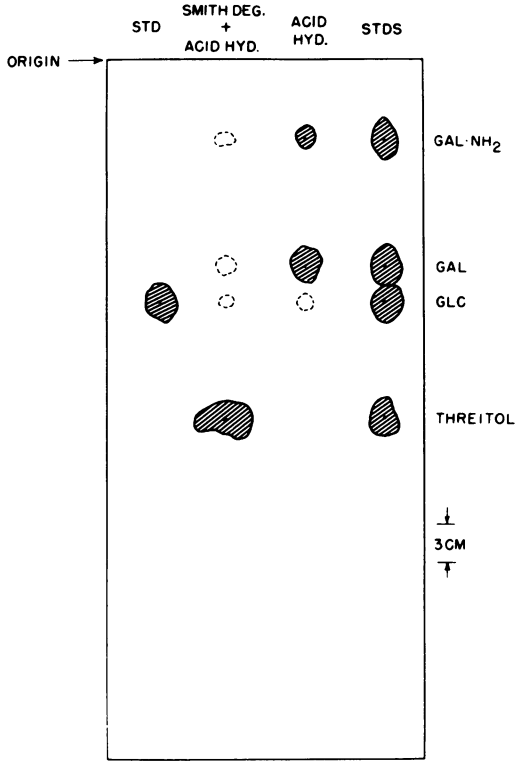

FIG. 9. *Paper chromatography (descending) of acid-hydrolyzed partially N-acetylated galactosaminogalactan (Acid Hyd.) and products generated after complete acid hydrolysis of periodate-oxidized and borohydride-reduced polymer (Smith Deg. + Acid Hyd.). Visualization was accomplished by silver nitrate dip. Designations are the same as indicated in the legend for Fig. 8, except threitol indicates* D*-threitol. Chromatography was conducted in solvent system 1 for 15 h.*

As an independent check on the identity of the hexoses of the polysaccharide that are resistant to periodate oxidation, a separate portion of Smith-degraded galactosaminogalactan (completely *N*-acetylated) was subjected to total acid hydrolysis and then reduced with [^{3}H]borohydride. Results obtained from paper chromatography in solvents 1, 2, and 3 indicated a major component of [^{3}H]galactosaminitol, plus minor fractions of [^{3}H]sorbitol and [^{3}H]galactitol. These results support those detailed above: galactosamine of completely *N*-acetylated polymer is resistant to periodate oxidation under the conditions employed.

Complete acid hydrolysis of polyalcohol from Smith-degraded partially *N*-acetylated galactosaminogalactan afforded glycerol and threitol in a molar ratio of 1:50, as determined by quantitative analysis of their acetates by gas chromatography. If one assumes that complete degradation affords 1 mol of glycerol from each end of a polymer chain, then the average number of anhydrohexose units is at least 100, since there are some resistant units.

Methylation analysis of galactosaminogalactan. Depolymerization of the permethylated polysaccharide yielded four components as revealed by a gas-liquid chromatographic analysis of their alditol acetates. Data for this study are presented in Table 6. Component III, one of two major constituents, was found to correspond to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol, identified by co-chromatography with an authentic compound. Its retention time relative to a standard of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol was also in agreement with that reported by Lindberg (30) but different from a standard of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl galactitol and from reported values for 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methyl and 1,3,4-tri-*O*-acetyl-2,4,6-tri-*O*-methyl derivatives (30). The minor tri-*O*-methyl component (II) was identified as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol by comparison with a reference standard. Authentic specimens of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol both had retention times greater than the tri-*O*-methyl glucitol component. The second major constituent of the methylated polysaccharide hydrolysate was identified as 2-deoxy-3,6-di-*O*-meth-

TABLE 6. *Gas-liquid chromatographic analysis of permethylated alditol acetates*[a]

Component	Relative retention time[b]	Relative peak area	Identity of acetate derivative
I	0.40	Trace	2,3,4,6-tetra-*O*-methyl galactitol acetate
II	0.72	Minor	2,4,6-tri-*O*-methyl glucitol acetate
III	1.00	Major	2,3,6-tri-*O*-methyl galactitol acetate
IV	4.2	Major	3,6-di-*O*-methyl 2-deoxy-2-*N*-methyl-acetamido galactitol acetate

[a] Analyses were conducted with a glass column (4 feet by 1/8 inch [110 by 0.31 cm]) with 3% ECNSS-M on Gas-Chrom P at 160 C. All four components were identified by co-chromatography with authentic standards.

[b] The relative retention time of the 2,3,6-tri-*O*-methyl galactitol acetate is 2.50 relative to standard 2,3,4,6-tetra-*O*-methyl-D-glucitol acetate.

yl-2-*N*-methylacetamido galactitol, also on the basis of co-chromatography with an authentic specimen (22). Furthermore, others (11, 52) have shown that the 4,6- and 3,4-di-*O*-methyl-*N*-methyl galactosaminitol acetates are well separated from the 3,6-di-*O*-methyl derivative on this column. Paper chromatography of a portion of the hydrolysate, before reduction and acetylation, was carried out with solvent system 7 (22), followed by detection with ninhydrin. The methylated amino sugar had a mobility identical to a sample of authentic 3,6-di-*O*-methyl-2-*N*-methyl-galactosamine hydrochloride. On the basis of this evidence, galactosamine units of the polysaccharide are linked (1→4) rather than (1→3) or (1→6), a conclusion consistent with the Smith degradation results reported above. The optical rotation of the permethylated polymer is indicative of a high percentage of α linkages in the polysaccharide.

DISCUSSION

The data presented in Table 1 and Fig. 2 and 3 identify the major component sugars of the polysaccharide as galactose and galactosamine and indicate that they occur in a molar ratio of approximately 7:2. Their enantiomeric configurations have not been determined. The fact that a minor glucose component is present in four different preparations of galactosaminogalactan strongly suggests it may be a constituent of the polysaccharide, although there is no additional evidence at present to support this. The resistance of polymer glucose to periodate oxidation and the *O*-methyl derivative of this hexose identified (Table 6) indicate glucose is substituted by 1,3 bonds. However, α- or β-1,3 glucans could also account for the results obtained since these polysaccharides are both constituents of *A. niger* hyphae (24, 51). It should be emphasized, however, that isolation of the galactosaminogalactan involves exhaustive extraction of tightly associated glucan with NaOH. Monitoring of the glucose content of the polysaccharide fraction by gas chromatography during fractionation is utilized to estimate purity. It has consistently been observed that this small percentage of glucose is not removed by exhaustive NaOH extraction.

Since direct extraction of the hyphal walls with 6 M guanidinium hydrochloride fails to liberate any detectable galactose, it is possible there exist alkali-labile linkages between galactosaminogalactan and another wall constituent, perhaps protein. Attachments between polysaccharide and protein occur in wall structure of both fungi (18) and yeast (46). If this is so, then liberation of galactosaminogalactan could occur during the purification procedure by alkaline cleavage of glycosyl-aminoacyl linkages.

Several experiments described in this study provide evidence that galactosaminogalactan is a copolymer of galactose and galactosamine. These include: (i) co-purification of these two sugars through the isolation procedure, (ii) co-elution of both saccharides in a constant ratio from porous glass bead columns with two different fractionation ranges (Fig. 6 and data not shown), and (iii) deaminative cleavage of the polysaccharide and reduction of the fragments with [^{3}H]borohydride, giving water-soluble products with a wide spectrum of molecular sizes (Fig. 7a and b). Since the chitosan control sample of equal weight was quantitatively cleaved to 2,5-anhydromannitol, as expected for a homoglycosaminoglycan, sufficient nitrous acid reagent was present to give complete cleavage of all hexosaminyl units present in galactosaminogalactan.

Further evidence that deaminative cleavage of the galactosaminogalactan is complete was obtained by the following experiment. An aliquot of peak I (Fig. 7b) was allowed to react with nitrous acid as described above. The elution profiles of the sample and a control were compared and found to be identical. All radioactivity of both samples eluted at the void volume of the 200-cm Bio-Gel P-2 column. There was no evidence of any further cleavage of the high-molecular-weight fraction by a second treatment of peak I with the diazotization reagents.

Acid hydrolysis (Fig. 4 and 5), Smith degradation (Fig. 8 and 9, Table 5), and methylation studies (Table 6) are all consistent with a pyranosyl structure for galactose. Threitol is derived from Smith degradation of 4-*O*-substituted galactopyranosyl and 5- or 6-*O*-substituted galactofuranosyl units, whereas arabinitol would be a product from 3-*O*-substituted galactofuranosyl moieties. The acid stability of polymer galactose (Fig. 5) plus the observation that it is almost completely destroyed by the Smith degradation are additional facts that argue strongly for a structure in which galactose is present in pyranose form and is substituted on its 4 position. Gas chromatographic analyses of methylated alditol acetates prepared from galactosaminogalactan are consistent with the other structural analyses and support (1→4) linkages for galactose and (1→3) linkages for glucose. The assignment of a 4-*O* substitution to the galactosamine residues is also based on results of both Smith degradation and methyla-

tion studies (Table 6). Hexosamine recovery from fully *N*-acetylated polysaccharide is not decreased by Smith degradation (Table 5, Fig. 8). However, degradation of partially *N*-acetylated galactosaminogalactan revealed significant destruction of galactosamine both by paper (Fig. 9) and gas chromatography. It was not possible to make meaningful calculations from gas chromatographic tracings in this latter experiment regarding its percentage of destruction since the galactosaminitol acetate peak detected after degradation was too small to be quantitated accurately. The qualitative results of this experiment are completely consistent with other observations, however, since complete destruction of hexosamine was never obtained by Smith degradation. Because 20% of polymer hexosamine is *N*-acetylated this percentage will be resistant to periodate even when 4-*O* substituted.

Periodate oxidation data also indicate some resistant units are present in the polysaccharide. Assuming that uptake of periodate by the nigeran control (92% of theoretical value) reflects a similar percentage of uptake by the galactosaminogalactan, then the true value for this polysaccharide would approximate 0.8 mol of periodate consumed per mol of anhydrohexose. The observed stoichiometry (less than 1 mol taken up per mol of anhydrohexose) probably reflects the sum of the glucose and *N*-acetylated galactosamine units in the polymer plus those periodate-resistant galactose moieties detected chromatographically after Smith degradation.

The polysaccharide reported here is of interest since it shows some similarities but many striking differences to the galactosaminogalactan identified in cultures of *A. nidulans* by Gorin and Eveleigh (20). Both are linear polymers composed of the same sugars in pyranosyl ring form and joined by α linkages. However, the *A. nidulans* polysaccharide is produced by submerged cultures and is exocellular, water soluble, and composed of galactose and galactosamine in a ratio of 1.8. Furthermore, its galactosamine is completely *N*-acetylated. Although the isolation procedures utilized in the present study could possibly cause some de-*N*-acetylation, we have found that direct diazotization and reduction of unfractionated hyphal walls results in the liberation of water-soluble tritiated polymeric material. Acid hydrolysis yields tritiated acetylated galactosamine is normally present in wall heteropolymer material.

Partially *N*-acetylated galactosamine polymers have been isolated from culture filtrates of *A. parasiticus* (13) and from *Neurospora crassa* hyphae (25, 32, 33) and culture filtrates (43). In all of these reports, isolation conditions preclude de-*N*-acetylation; therefore it is possible that the degree of *N*-acetylation somehow reflects a physiologically significant property of these molecules (28).

It is also possible that hexosamine may confer irregularity to the polysaccharide structure. X-ray diffraction patterns of *A. niger* galactosaminogalactan (kindly obtained by Robert Marschessault of the University of Montreal) indicate a noncrystalline structure for the polymer chains. It may be speculated that the random interruption of the galactopyranosyl repeating sequence by galactopyranosylamine units confers enough irregularity to prevent the interchain orientation that permits crystallization. A general role of hyphal polysaccharides containing galactosamine is unknown but at least one study suggests a possible physiological function. Reissig and Glasgow (43) have implicated the galactosamine polymer of *N. crassa* in control of its own growth rate. A mutant with a restricted growth character as well as wild-type cells in stationary phase was found to have this polysaccharide in its hyphal architecture. Furthermore, although it is normally partially *N*-acetylated, complete *N*-acetylation was found to abolish its regulatory activity. However, somewhat contradictory observations were made by Gratzner (23), who found that starved *N. crassa* hyphae have lower amounts of galactosamine than those undergoing rapid vegetative growth. Decreased hyphal galactosamine associated with increasing culture age has also been noted with *A. parasiticus* (13).

Although the ubiquitous distribution of galactopyranose and furanose units in fungal and yeast polysaccharides is now clearly established, the great diversity in structures of these molecules suggests a variety of roles wherein galactose may confirm some particular functional characteristics in the polysaccharides. One major group includes the neutral galactans and galactomannans such as those found in *Trichophyton* species (3, 5, 6, 9) and *Penicillium chrysogenum* (37). The polysaccharides of a second class contain galactosyl moieties, but in addition each has charged groups as part of its carbohydrate structure. Examples include galactosamine polymers of *Aspergillus* and *Neurospora*, the phosphogalactomannan of *P. charlseii* (18), and the phosphogalactan of *Sporobolomyces* yeast (48), which has phosphate esterified in position 6 of galactopyranosyl residues. The galactosaminogalactans ap-

pear to display characteristics of both classes.

Although resolution of the fine structure of the polysaccharide obviously awaits further chemical characterization of the deaminative cleavage fragments and determination of their relative proportions in the molecule, the gel filtration profiles observed with ^{3}H-reduced fragments (Fig. 6a and b) indicate that many non-*N*-acetylated galactosamine units are separated by varying (probably random) intervals of nonreactive saccharides. A polysaccharide with a regular repeating unit would yield one major labeled fragment (31). Indeed, the data of Gorin and Eveleigh (20) support a structure for the *A. nidulans* polysaccharide that has a regular trisaccharide repeating sequence of two galactose and one *N*-acetyl galactosamine residues. The galactosaminogalactans described in the present study and previously (20) offer a possible new class of polysaccharides of general occurrence in fungi. Interestingly, they contain no mannose; a majority of mycelial polymers containing galactose are galactomannans and phosphogalactomannans (17, 21) and tend to be quite highly branched (3, 5, 6, 9, 18, 34, 43).

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