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# Characterization of a Cytoplasmic Reserve Glucan from *Ruminococcus albus*

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*Ruminococcus albus*, an anaerobic bacterium that digests cellulose in the rumen of cattle, produces intracellular polysaccharide granules varying from 0.05 to 0.31 μm in diameter when grown in batch culture. This polysaccharide material was purified and found to contain D-glucose as the only reducing sugar. The polyglucose polymer was slightly opalescent in aqueous solution and formed a strong reddish purple iodine complex with a maximum absorbance at 550 nm. Its infrared spectrum had characteristic absorption bands at 8.70, 9.25, and 9.75 μm and was identical with that of the amylopectin-glycogen type of *Megasphaera elsdenii* and that of the glycogen of enteric bacteria and beef liver. It reacted strongly with concanavalin A. Methylation analysis showed that the glucan contained 2,3,4,6-tetra-*O*-MeG–2,3,6-Tri-*O*-MeG–2,3-Di-*O*-MeG in a ratio of 8:84:8. Characterization of the products obtained by treatment with isoamylase indicates that the glucan of *R. albus* is of the glycogen type.

Reserve glucans containing predominantly α-(1 → 4) linkages frequently occur in bacteria, particularly in anaerobic organisms (3, 5, 31). Electron microscopy of the rumen contents of cows fed on a fine-particle size, high-energy diet revealed that a large portion of a mixed population of rumen bacteria contained intracellular granules varying in size from 0.05 to 0.30 μm (5). A reserve glucan from *Megasphaera elsdenii*, one of the rumen bacteria implicated in feedlot bloat (9), has been purified and characterized as an amylopectin-glycogen type according to the revised Meyer-Bernfeld model (2, 5, 7).

This study was undertaken to determine the nature of the intracellular polysaccharide granules (15) formed by *Ruminococcus albus*, an important digester of cellulose in the rumen.

## MATERIALS AND METHODS

**Organism and culture conditions.** *Ruminococcus albus* (B199) was generously provided by M. P. Bryant, University of Illinois, Urbana, Ill.

The anaerobic technique used throughout this investigation for culturing these rumen bacteria was essentially that of Hungate (14) as modified by Bryant and Burkey (4). Cultures of *R. albus* that had been grown anaerobically for 10 h at 39°C in prereduced artificial medium containing 0.5% glucose or cellobiose (28) on a rotary shaker (75 rpm) were inoculated into 300 ml of the prereduced artificial medium (5% vol/vol) in round-bottom flasks (500 ml) and further incubated for 28 h with shaking. At 2-h intervals up to 28 h after inoculation, cultures were fixed and embedded for electron microscopy (5) or harvested by centrifugation in a Sorvall RC-2B refrigerated centrifuge for 10 min at 15,000 × *g* for glucan extraction.

**Chemicals.** Tris(hydroxymethyl)aminomethane, oyster glycogen, rabbit liver glycogen, beef liver glycogen, β-amylase (EC 3.2.1.2), and α-amylase (EC 3.2.1.1) were purchased from the Sigma Chemical Co., St. Louis, Mo. Osmium tetroxide and Vestopal W were purchased from Polysciences Inc., Rydal, Pa. Trimethysilylated sugar and TRI-SIL "Z" for rapidly preparing trimethylsily derivatives of sugar were purchased from Chromatographic Specialties, Brockville, Ontario. Glucan from *M. elsdenii* was obtained and purified as described previously (2, 5). Isoamylase was obtained from Senn Chemicals, Dielsdorf, Switzerland.

**Polysaccharide isolation and purification.** Early stationary-phase cells of *R. albus* (10 h; optical density, 1.2 to 1.3 absorbancy units) were harvested, washed twice with glass-distilled water, and freeze-dried. A 5-g portion of dried cells was suspended in 0.1 M tris(hydroxymethyl)aminomethane (100 ml) containing 0.1 M ethylenediaminetetraacetic acid and 1 mg of lysozyme per ml (pH 8.4) and incubated at 37°C on a shaker for 2 h. The cells were disrupted for three 30-s intervals with a Bronson sonifier at a tip energy of 100 W. Cell breakage was observed by phase microscopy. The suspension was transferred to a flask (250 ml) and an equal volume of 30% NaOH was added (11). This mixture was hydrolyzed for 3 h in a boiling-water bath. The solution was centrifuged at 48,000 × *g* for 30 min; the supernatant fluid was retained, and the pellet was discarded. Two volumes of cold 95% ethanol were slowly added to the supernatant at 5°C to precipitate the polysaccharide. This mixture was stored in the refrigerator (4°C) overnight and then centrifuged at 48,000 × *g*

for 20 min. The supernatant was discarded, and the white precipitate was suspended in slightly acidic water and reprecipitated with two volumes of 95% ethanol. The white precipitate was dissolved in water and dialyzed against glass-distilled water for 6 days at 4°C. The solution remaining in the dialysis tube was freeze-dried to yield a white powder, which was dissolved in slightly acidified distilled water and loaded onto a Sephadex G-200 column (bed volume, 5 by 85 cm) previously equilibrated with distilled water. The column was eluted at a flow rate of 6.7 ml/h with distilled water, and 10-ml fractions were collected. The polysaccharide appeared in the fractions corresponding to the void volume. These fractions were pooled and lyophilized to yield fraction I.

Fraction I was further purified by precipitation with low-alkaline copper reagent (12). Two fractions (fraction II, insoluble; fraction III, soluble) were collected, salts were removed, and portions of both fractions were methylated. Fraction II was chromatographed using a column (2.5 by 50 cm) of Sepharose 4B, which had been developed with water at a flow rate of 4 ml/h. Fractions (4 ml) were collected, and material from the first peak was lyophilized to yield fraction IV.

**Digestion of glucan by α-amylase, β-amylase, or saliva.** Saliva was obtained from a rumen-fistulated cow. Glucans from the cells of *M. elsdenii* and *R. albus*, oyster and beef liver glycogen, and amylopectin were dissolved in 0.01 M phosphate buffer (pH 7.0) containing 0.0125 M NaCl. The glycogen suspension was incubated with α-amylase, β-amylase, or saliva at 37°C for 24 h (17), and changes in opalescence and coloration with iodine were observed.

**Total carbohydrate determination.** The total carbohydrate concentration in the isolated polysaccharide of *R. albus* was determined by the anthrone procedure of Neish (20), which used glucose as the standard.

**Glucose determination.** To determine if the polysaccharide was a polyglucose polymer, 2 to 10 mg of purified material was hydrolyzed under vacuum in 1 N HCl or 1 N $H_2SO_4$ at 100°C for 18 h. The hydrolysate was neutralized with 1 N NaOH. The glucose concentration of the hydrolyzed polysaccharide was determined by the Worthington glucostat procedure (30), and absorbance was read at 420 nm on a Gilford 300-N spectrophotometer. Glucose concentration was proportional to optical density over a concentration range of 0 to 50 μg/ml.

Samples (5 μl) of the hydrolysate and sugar standards were spotted on Whatman no. 1 filter paper. The chromatograms were developed by a descending flow with the upper layer of butanol-acetic acid-water (4:1:5). Aniline phthalate (21) was used to locate the sugar spots. Ninhydrin spray was used to locate ninhydrin-positive materials (27).

**Isoamylase hydrolysis of glucan.** Fraction IV and oyster glycogen were treated with isoamylase as described by Gunja-Smith et al. (8), and the products were chromatographed on a column (1 by 30 cm) of Sephadex G-75, which had been developed with water at a flow rate of 1 ml/h. Fractions (1 ml) were collected and analyzed for total carbohydrate.

**Methylation analysis.** Samples were methylated using the Hakomori procedure (10), hydrolyzed, and converted to alditol acetate derivatives, which were analyzed by gas-liquid chromatography using the following columns: (i) 3% ethylene-succinate-cyanoethylsilicone copolymer fluid on Gas-Chrom Q (100 to 120 mesh) at 165°C; (ii) 3% cyanopropylmethylphenylmethylsilicone fluid (OV-225) on Gas-Chrom Q (100 to 120 mesh) at 175°C; and (iii) SP1000 glass capillary column (0.25 mm by 25 m; LKB, Bromma, Sweden) at 240°C. For mass spectrometry, a Perkin-Elmer 270 combination gas chromatograph-mass spectrometer was used.

**Formation of insoluble complex with concanavalin A.** For the turbidimetric determination of glucan-concanavalin A complex, 9 ml of the concanavalin A (9 mg) solution was added to 1 ml of solution containing 0.1 to 1.0 mg of glucans. After 15 min at room temperature, the optical density at 420 nm was measured on a Gilford model 300-N spectrophotometer against a concanavalin A-water control and glucan-water control (6, 19).

**Spectrometry.** The absorption spectrum of the polysaccharide-iodine complex was recorded in the visible spectrum between 400 and 700 nm using a Cary spectrophotometer. A 1.0-ml solution containing a final concentration of 0.03% purified polysaccharide material and 0.02% iodine in 0.20% aqueous KI was measured spectrophotometrically against an iodine-iodide reference solution containing no polysaccharide (22). Infrared spectra were measured with a Perkin-Elmer model 21 spectrophotometer in KBr pellets (1 mg/400 mg of KBr).

**Electron microscopy.** Glutaraldehyde (5%) in 0.02 M sodium potassium phosphate buffer (pH 6.2) was used as the fixative. The anaerobically cultured cells were prefixed by adding 1 volume of the fixative to 9 volumes of the cell culture and holding for a minimum of 15 min at room temperature. The cell suspensions were centrifuged, and the pellets were resuspended in the fixative for 2 h. The suspensions were centrifuged, and the pellets were enrobed in 3% agar. The agar cores were washed five times (10 min) in veronal-acetate buffer (26) and postfixed for 1 h in 2% osmium tetroxide in veronal-acetate buffer containing $7 \times 10^{-3}$ M $MgCl_2$ and 0.16% tryptone. The agar cores were washed five times (10 min) with veronal-acetate buffer, dehydrated by 30-min passages through increasing concentrations of acetone (30, 50, 70, 90, 95, and 100%) and propylene oxide (100%), and embedded in Vestopal W. Thin sections were cut with a Porter-Blum MT-2 ultramicrotome and attached to clean 400-mesh copper grids. The sections were stained with 1% uranyl acetate at pH 5.0 and lead citrate (24) and examined by means of an AEI EM-801 electron microscope with a 60-kV electron-acceleration voltage.

## RESULTS

**Formation of granules.** The formation of polysaccharide granules during the growth of *R. albus* in batch culture was followed by examination of thin sections of the cells by electron microscopy. In a 4-h culture, most of the cells contained no polysaccharide granules, but

some cells had a few granules. In late log-phase cultures, the number of cytoplasmic polysaccharide granules increased, and an 8-h culture showed this accumulation (Fig. 1A). As the cultures reached stationary phase at 10 h, the cells became packed with polysaccharide granules (Fig. 1B). Cells of 14-h cultures contained small numbers of polysaccharide granules, and some cells contained no granules (Fig. 1C). Some of the polysaccharide granules were enclosed by a single electron-dense layer (Fig. 1A, arrow). The normal nucleoid and particulate cytoplasmic elements were often displaced to the cell periphery by the presence of these carbohydrate deposits (Fig. 1B).

**Properties of the crude polysaccharide preparation.** Aqueous solutions of the polysaccharide preparation isolated from early stationary-phase cultures of *R. albus* had a strong opalescence that disappeared when NaOH was added. They gave a strong reddish purple color with iodine and had no reducing activity before hydrolysis. Treatment with saliva or $\alpha$-amylase rapidly removed the opalescence, decreased the capacity for coloration with iodine, and caused the appearance of reducing power, which suggested the presence of $\alpha\{1 \rightarrow 4\}$-glucosidic linkages (Table 1). The absorption spectra of the iodine complex of the polysaccharide had a peak at 545 to 550 nm compared with glucan from *M. elsdenii*, which had a peak at 490 to 495 nm (Fig. 2).

The infrared spectrum was identical to that of the glucan of *M. elsdenii*, enteric bacteria (18), oyster, rabbit liver, and beef liver and had the respective characteristic absorption bands of these glucans at 8.7, 9.25, and 9.75 $\mu$m (Fig. 3). Chromatography of a hydrolysate revealed a single component with an $R_f$ value identical to that of glucose (Table 2). No ninhydrin-positive materials were detected on the paper chromatograms. Gas-liquid chromatography also provided verification that glucose is the only carbohydrate component (Table 2).

Yields of crude glucans by NaOH extraction and ethanol precipitation of cells grown on glucose or cellobiose media were 0.268 and 0.865 g, respectively, from a 5-g portion of dried cells. The crude glucans were found to be 51% glucose for glucose-grown cells and 64% for cellobiose-grown cells according to the anthrone method of glucose determination. This gave a corrected glucan yield of about 2.73% of cell dry weight for glucose-grown cells and 11.07% for cellobiose-grown cells. The crude glucans were further purified by chromatography on Sephadex G-200. Much of the polysaccharide appeared in the void volume, indicating a molecular weight greater than 200,000. Hydrolysis of this purified glucan showed that it contained 84.8 to 89.6% glucose by both the glucostat procedure and the anthrone method.

**Structural studies.** Methylation analysis of fraction I indicated the presence of 4-*O*- and 4,6-di-*O*-substituted D-glucopyranose residues (Table 3). In addition, an unknown compound, which mass spectrometry indicated was not a sugar derivative, was present. Copper precipitation produced two fractions. In a typical separation, a 50.2-mg portion of fraction I yielded 17.2 mg of fraction II and 26.2 mg of fraction III.

Only the soluble component (fraction III) contained the unknown compound. Fraction II was purified by column chromatography (Fig. 4), and then the purified polysaccharide was treated with isoamylase. Gel filtration of the enzymic hydrolyzate (Fig. 5) demonstrated a distribution of products similar to that obtained from oyster glycogen.

The precipitation of glucans from anaerobic rumen bacteria, glycogens, and amylopectins by concanavalin A (a globulin from jack bean meal) is shown in Fig. 6. The glucans of *R. albus* reacted as strongly with concanavalin A as did glucan from *M. elsdenii*. The glucan from beef showed similar ranges of values as that of glucans from *R. albus* and *M. elsdenii*, but amylopectin gave very low values.

## DISCUSSION

Batch cultures of *R. albus* showed that actively growing cells contained some cytoplasmic polysaccharide inclusions and that early stationary-phase cells accumulated so much of this material that the normal cytoplasmic components were displaced to the periphery of the cell. In contrast, late stationary-phase cells contained few or no polysaccharide inclusions. Hungate (15) also reported that, on treatment with iodine, the early stationary-phase cells of *R. albus* showed reddish violet to purple color and late log-phase cells produced a purple-black color; however, he reported that cells from old batch cultures gave no color. These results differ from those for another rumen bacterium, *M. elsdenii*, which showed that actively growing cells contained a few cytoplasmic polysaccharide inclusions, whereas old cells were filled with polysaccharide granules (5).

In most bacteria, polysaccharide granules accumulate when growth is limited by a deficiency in some vital substrate other than the carbon and energy sources (13, 23, 29, 32). The accumulation of polysaccharide by *R. albus* occurs in the late log and early stationary phases. Although the exact purpose of this accumulation in *R. albus* is not known, we suspect that the polysaccharide granules serve as an intra-

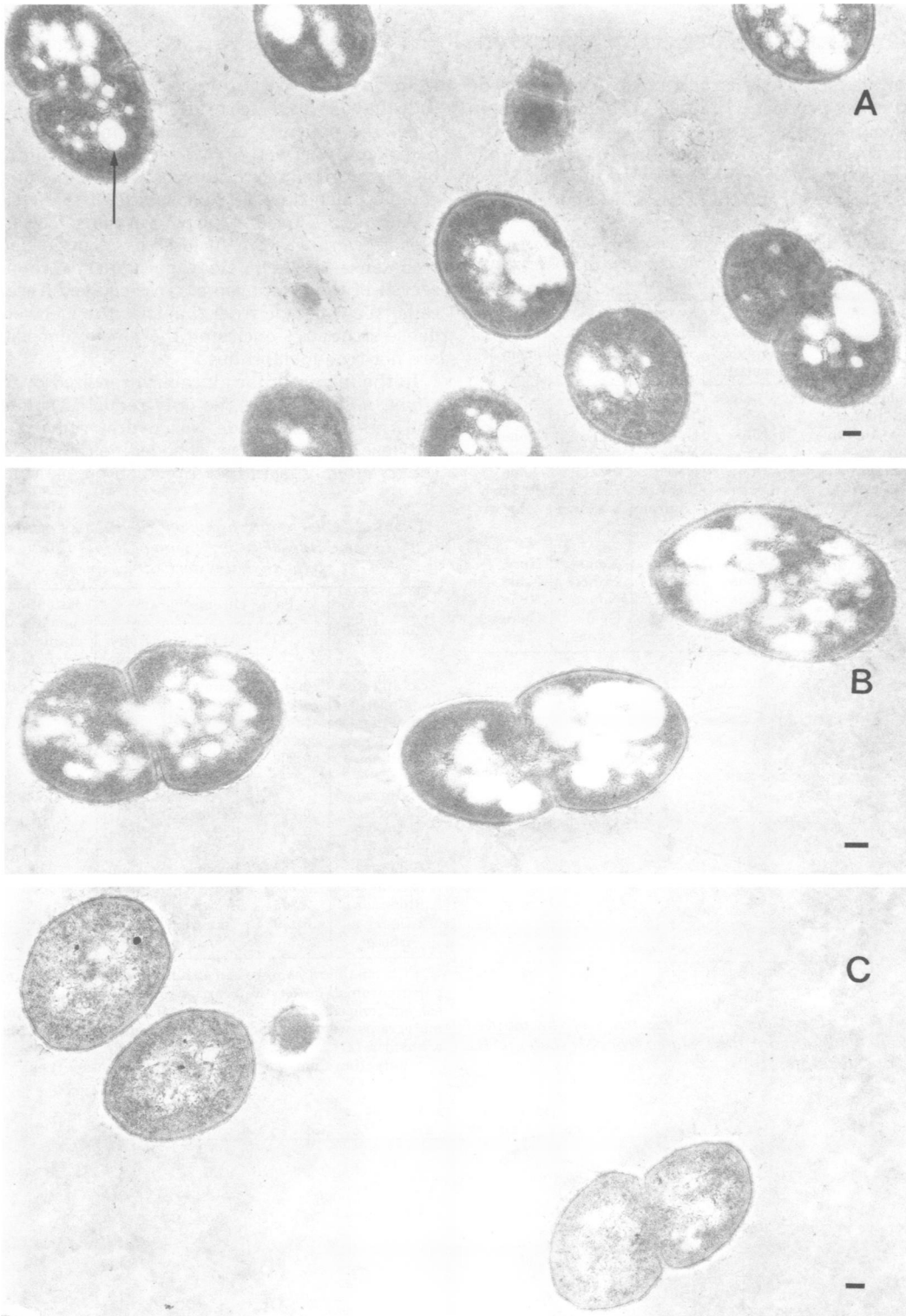


FIG. 1. *Electron micrograph of cells from a culture of R. albus. The bar indicates 0.1 μm. (A) Late log phase (8 h) showing that some cells contain large numbers of cytoplasmic inclusions. Note the single electron-dense layer (arrow) bounding these inclusions. (B) Early stationary phase (10 h) showing a massive accumulation of cytoplasmic inclusions in all cells. The normal cytoplasmic contents of the cell are displaced to a peripheral area, and the cells appear to be completely full of inclusions in some section planes. (C) Late stationary phase (14 h) showing that some cells contain a few cytoplasmic inclusions, whereas most cells did not contain any inclusions.*

cellular reserve of carbon and energy as they do in other bacteria (1, 25, 32). The difference in glucan storage between glucose- and cellobiose-grown cells (2.73 versus 11.00% of cell dry weight) may be explained by the observation of Iannotti et al. (16) that cells of another strain of *R. albus* transport glucose very slowly, i.e., they have a high saturation constant. Moreover, the splitting of cellobiose to hexose by phosphorolytic activity, to provide building blocks for glucan synthesis, conserves one molecule of adenosine 5'-triphosphate.

Although some of the polysaccharide granules in *R. albus* were enclosed by a single electron-dense layer, no cleavage planes were observed in the cytoplasm of cross-cleaved frozen cells. We conclude from this that the electron-dense structures enclosing the glucan deposits are not true membranes.

In the intracellular glucan synthesized by *R. albus*, D-glucose was the only reducing sugar, and the polysaccharide was hydrolyzed by α-amylase and isoamylase. The iodine complex of the *R. albus* glucan had a reddish purple color

TABLE 1. *Digestion of glucans by α-amylase, β-amylase, or saliva and the changes in opalescence and coloration with iodine*

| Treatment | Glucans | | | |
|---|---|---|---|---|
| | Amylopectin | From *R. albus* | Beef glycogen | From *M. elsdenii* |
| Coloration with iodine | | | | |
| α-Amylase | None | None | None | None |
| β-Amylase | None | None | None | None |
| Saliva | None | None | None | None |
| Control | Purple | Reddish purple | Reddish brown | Reddish brown |
| Opalescence | | | | |
| α-Amylase | None | None | None | None |
| β-Amylase | None | None | None | None |
| Saliva | None | None | None | None |
| Control | Opalescence | Opalescence | Opalescence | Opalescence |

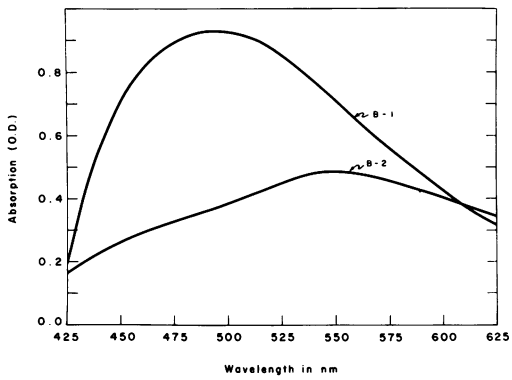


FIG. 2. *Light absorption spectrum of the iodine-stained polysaccharides isolated from M. elsdenii (B-1) and R. albus (B-2).*

TABLE 2. *Chromatographic characteristics of the acid-digested intracellular glucan of R. albus and its trimethylsilyl derivatives*

| Compound | Paper chromatogram[a] | | | Retention time of gas chromatogram (s)[b] |
|---|---|---|---|---|
| | $R_f$ | Aniline phthalate | Ninhydrin | |
| Acid-digested glucan | 0.152 | Brown | None | 212 and 302 |
| Standards | | | | |
| Ribose | 0.276 | Reddish | None | 56 |
| Arabinose | 0.200 | Reddish | None | 94 |
| Xylose | | | | 127 |
| Mannose | | | | 186 |
| Fructose | 0.202 | Yellowish | None | 193 |
| α-Glucose | 0.152 | Brown | None | 212 |
| Sorbose | | | | 212 |
| Galactose | 0.142 | Brown | None | 222 |
| β-Glucose | 0.152 | Brown | None | 302 |
| Rhamnose | 0.345 | Brown | None | |
| 2-Deoxy-D-ribose | 0.440 | Yellowish | None | |

[a] Chromatograms were run 48 h in this case. Descending paper chromatography was carried out with upper layer of solvent composed of butanol-acetic acid-water (4:1:5). Chromatograms were sprayed with aniline phthalate (21) and ninhydrin (27).

[b] Retention times were measured at the center of peak.

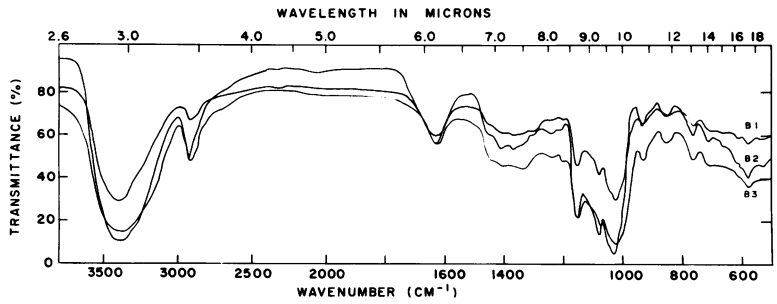


FIG. 3. *Infrared absorption spectra (KBr pellets) of polysaccharides from M. elsdenii (B1), glycogen from beef liver (B2), and polysaccharides from R. albus (B3).*

TABLE 3. *Methylation analysis of the glucan fractions from R. albus glucan*

| Sugar[a] | Tg column[b] | | | Molar proportions (%) | | |
|---|---|---|---|---|---|---|
| | ECNSS-M | OV-225 | SP1000 | Fraction I | Fraction II | Fraction III |
| 2,3,4,6-Tetra-*O*-MeGlc | 1.00 | 1.00 | 1.00 | 6 | 8 | 12 |
| Unknown | 2.45 | 1.75 | 1.83 | 48 | 0 | 33 |
| 2,3,6-Tri-*O*-MeGlc | 2.45 | 2.30 | 1.98 | 39 | 84 | 49 |
| 2,3-Di-*O*-MeGlc | 5.39 | 4.50 | 3.20 | 5 | 8 | 4 |

[a] Glc, D-Glucose

[b] Tg is the retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations for columns: ECNSS-M, 3% ethylenesuccinate-cyanoethylsilicone copolymer fluid on Gas-Chrom Q (100 to 120 mesh) at 165°C; OV-225, 3% cyanopropylmethyl-phenylmethylsilicone fluid on Gas-Chrom Q (100 to 120 mesh) at 175°C; SP1000, glass capillary column (LKB, Bromma, Sweden) at 240°C.

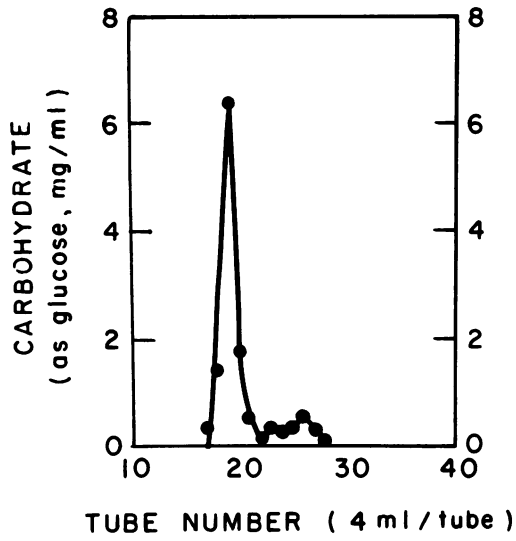


FIG. 4. *Gel filtration of fraction II on a column of Sepharose 4B. A 42-mg yield (tubes 17 through 21) from 65 mg placed on the column was obtained.*

and maximum absorption at 550 nm compared to reddish brown and 493 nm for *M. elsdenii* glucan. The infrared spectrum of the glucan from *R. albus* with characteristic absorption bands at 8.70, 9.25, and 9.75 μm was identical to that of the glucan of *M. elsdenii* and beef glycogen. The glucans from *R. albus*, *M. elsdenii*, and beef glycogen reacted more strongly with concanavalin A than with the amylopectin. An almost linear relationship was found between the concentration of glucans from *R. albus* or *M. elsdenii* or the glycogen from beef and the resultant turbidity after treatment

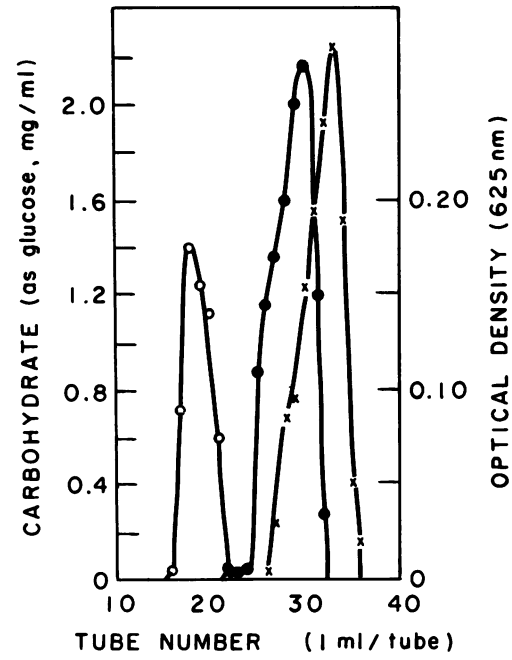


FIG. 5. *Gel filtration of enzymic hydrolysates of R. albus glucan and oyster glycogen on a column of Sephadex G-75. Symbols: ○, Blue dextran (measured by absorption at 625 nm); ●, R. albus hydrolysate; x, oyster glycogen hydrolysate.*

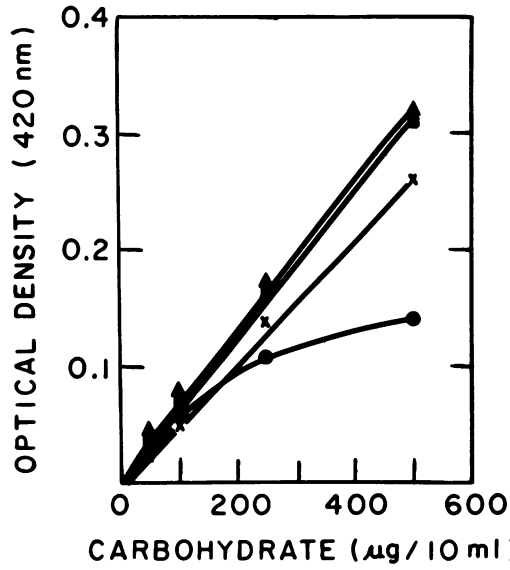


FIG. 6. *Formation of insoluble complex between glucans and concanavalin A. A 9-ml portion of concanavalin A (1 mg/ml) solution was added to 1 ml of solution containing 0.1 to 0.5 mg of glucans. Symbols: ●, Amylopectin; ▲, glucan from R. albus; ■, glucan from M. elsdenii; x, beef glycogen.*

with concanavalin A. Glucans from *R. albus* and from *M. elsdenii* appear to have similar structures as revealed by methylation analysis, enzymic hydrolysis with isoamylase, and precipitation by concanavalin A (2).

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