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Composition and Structure of the O-Specific Side Chain of Endotoxin from *Serratia marcescens* 08†

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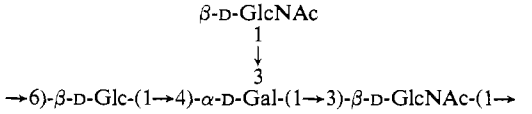
ABSTRACT: The endotoxin complex of *Serratia marcescens* 08 was hydrolyzed by 1% acetic acid, and the O-specific side chain was isolated from the hydrolysate by ion-exchange chromatography on Dowex 1 and gel filtration on Sepharose 4B. The determinations of chemical composition and molecular weight indicated that the purified O-specific side chain was a polysaccharide consisting of D-glucose, D-galactose, and *N*-acetyl-D-glucosamine in a molar ratio of 1:1:2. On the basis of evidence obtained from periodate oxidation, methylation, mass spectrometry, infrared spectroscopy, hydrazinolysis, and partial acid hydrolysis, it was concluded that the O-specific side chain is a polysaccharide consisting of repeating units of a branched tetrasaccharide. The average number of repeating units in the O-specific side chain was estimated to be 17. The structure and anomeric configuration of the repeating unit were identified as



Recently, we demonstrated (Wober and Alaupovic, 1971; Wang and Alaupovic, 1973) that the polysaccharide moieties of endotoxins from a chromogenic and a nonchromogenic strain of *Serratia marcescens* consist of the same structural entities found in *Salmonella* (Lüderitz, 1970), *Escherichia* (Heath *et al.*, 1966; Schmidt *et al.*, 1969), and *Shigella* (Simmons, 1969). To isolate and identify these entities, we hydrolyzed the endotoxins with 1% acetic acid and separated the resulting partially degraded polysaccharide moieties ("degraded polysaccharides") by dialysis or by Sephadex gel filtration into two fractions. These corresponded to the O-specific side chain and oligosaccharide core, respectively. Further studies (Wang and Alaupovic, 1973) revealed that the O-specific side chain from the nonchromogenic strain *S. marcescens* Bizio is a linear polysaccharide which contains as a repeating unit a D-glucose–L-rhamnose disaccharide. This work represented the first structural elucidation of an O-specific side chain from the genus *Serratia* and provided evidence that even disaccharides may form repeating units.

The results presented in this article show that, in contrast to the disaccharide repeating unit of the nonchromogenic strain, the O-specific side chain of the chromogenic strain *S. marcescens* 08 consists of repeating units of a branched tetrasaccharide.

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Materials and Methods

Authentic Sugars. The 2,3,4-tri-*O*-methyl-D-glucose was obtained from D-raffinose, the 2,3,4,6-tetra-*O*-methyl-D-glucose from panose, and the 2,3,4,6-tetra-*O*-methyl-D-galactose from lactose. These commercially available oligosaccharides (Mann Research Laboratories, New York, N. Y.) were reduced with sodium borohydride and permethylated. The methylated oligosaccharides were then hydrolyzed, reduced, and acetylated as described by Björndal *et al.* (1970). The resulting alditol acetates were separated by gas–liquid chromatography and characterized by their relative retention times. The identities of 2,3,4-tri-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose were also verified by gas–liquid chromatography and mass spectrometry. D-Erythritol was purchased from Mann Laboratories, New York, N. Y. D-Threitol was prepared according to the method of Barnett and Kent (1963).

Bacteria. Cells of the chromogenic strain *Serratia marcescens* 08, cultivated and harvested as described previously (Alaupovic *et al.*, 1966), were supplied by General Biochemicals (Chagrin Falls, Ohio).

Isolation and Purification of the O-Specific Side Chain. To remove free lipids the nucleic acid free endotoxin isolated by trichloroacetic acid extraction of wet cells of *S. marcescens* 08 (Wober and Alaupovic, 1971) was extracted by chloroform–methanol (2:1, v/v) in a Soxhlet apparatus for 24 hr. This endotoxin preparation was hydrolyzed with 1% acetic acid for 4 hr at 90° according to the method of Morgan and Partridge (1941). The flocculating precipitate, representing conjugated protein, was separated from the soluble "degraded polysaccharide" according to a previously described procedure (Wober and Alaupovic, 1971). The solution containing "degraded polysaccharide" was extracted by shaking with chloroform three times and the aqueous phase was lyophilized. The lyophilized residue was dissolved in distilled water, after which the solution was centrifuged at 105,000*g* for 3 hr to remove traces of intact endotoxin. To separate the O-specific side chain from the oligosaccharide core, 300 mg of lyophilized supernate was dissolved in 3 ml of distilled water and applied to a Dowex 1-X8 column (2.5 × 20 cm), 200–400 mesh, in bicarbonate form. The column was eluted with 100 ml of distilled water. The eluate, containing the O-specific side chain, was dialyzed exhaustively against distilled water and lyophilized. The O-specific side chain was recovered as a colorless, fluffy material, the yield being 70% of the "degraded polysaccharide" fragment. The oligosaccharide core was eluted from the column with 200 ml of 0.5 N ammonium bicarbonate. The O-specific side chain was further purified by Sepharose 4B column (2.5 × 100 cm) chromatography. Approximately 100 mg of the substance was dissolved in 3 ml of phosphate buffer (0.15 NaCl in 0.05 M phosphate buffer, pH 7.0). The column was eluted at a flow rate of 16 ml/hr and the fractions of 3 ml were collected. The elution pattern was monitored by determining the carbohydrate content of 0.1-ml aliquots of each fraction by the phenol–sulfuric acid method. The elution volume of the O-specific side chain was 303 ml. The void volume as determined by Blue Dextran was 165 ml.

Analytical Methods. The O-specific side chain and its oligosaccharide fragments were hydrolyzed with 1 N H_2SO_4 in evacuated, sealed glass tubes for 4 hr at 100°. The hydrolysates were neutralized with Dowex 1 (HCO_3^- form). D-Glucose was determined by the D-glucose oxidase method (Huggett and Nixon, 1957) and D-galactose by the D-galactose oxidase method (Fischer and Zapf, 1964), using the standard Glucostat and Galactostat reagents (Worthington Biochemical Corp., Freehold, N. J.). Reducing sugars were determined by the method of Park and Johnson (1949). The carbohydrate content of chromatographic fractions was determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956). D-Glucose and D-galactose were also estimated by gas–liquid chromatography after their conversion into alditol acetates according to the method of Sawardeker *et al.* (1965). D-Glycerol and D-threitol were identified as acetates by gas–liquid chromatography. Free amino groups were determined with dinitrofluorobenzene (Ghuysen *et al.*, 1968). D-Glucosamine was determined by the method of Rondle and Morgan (1955) and by the amino acid analyzer; D-glucosamine was differentiated from D-galactosamine by its specific elution volume from the Beckman Custom Spherical Resin Type PA-28 used for the analysis of acidic and neutral amino acids. Amino acid and fatty analyses were performed as described previously (Wober and Alaupovic, 1971).

Enzymatic Hydrolysis of Oligosaccharides. A. ENZYMES. Jack bean β-galactosidase (1.25 units/5 μl) with a specific activity of 30 units/mg of protein, jack bean β-*N*-acetylhexosaminidase (4.5 units/5 μl) with a specific activity of 102 units/mg of protein, and fig α-galactosidase (0.12 unit/μl) with a specific activity of 1.9 units/mg of protein were prepared according to previously reported procedures (Li and Li, 1968, 1970; Hakomori *et al.*, 1971). Enzyme units and specific activities were also defined in these reports. All the enzymes were free from other contaminating glycosidases. The β-glucosidase (1 mg/ml of water) was purchased from Sigma Chemical Co., St. Louis, Mo.

B. INCUBATION OF OLIGOSACCHARIDES WITH GLYCOSIDASES. Aliquots (50 μl) of oligosaccharide solutions (~0.1 μmol/50 μl) were evaporated to dryness and the residues dissolved in 100 μl of 0.05 M sodium citrate buffer, pH 4.0. These solutions were then incubated with 5 μl of the appropriate glycosidase (β-galactosidase, α-galactoside, and β-*N*-acetylhexosaminidase) for 2 hr at 37°. After incubation, the reaction mixture was applied to an automatic sugar analyzer for the determination of liberated D-galactose (Lee *et al.*, 1969). Free *N*-acetyl-D-glucosamine was determined by the method of Reissig *et al.* (1955). Oligosaccharides were hydrolyzed by β-glucosidase as described previously (Wang and Alaupovic, 1973).

Permethylation of the O-Specific Side Chain. The O-specific side chain was methylated with methylsulfinyl carbanion and methyl iodide in methyl sulfoxide (Hakomori, 1964). The permethylated polysaccharide was then hydrolyzed with 90% formic acid and 0.25 N H_2SO_4 at 100° and the methylated sugars were reduced and acetylated as described by Björndal *et al.* (1970).

Smith Degradation of the O-Specific Side Chain. The O-specific side chain (100 mg) was oxidized with 0.05 M sodium metaperiodate (50 ml) for 24 hr in the dark at room temperature according to the procedure described by Goldstein *et al.* (1965). The excess periodate was destroyed by treating the reaction mixture with 1 ml of ethylene glycol for 1 hr at room temperature. After reduction with sodium borohydride (600 mg) overnight at 4°, the solution was neutralized with acetic acid and desalted by gel filtration on a Sephadex G-15 column (2.5 × 100 cm). The lyophilized oxidation product was hydrolyzed with 0.5 N HCl for 7 hr at 37°, and the hydrolysate was lyophilized.

Hydrazinolysis of the O-Specific Side Chain. The O-specific side chain (500 mg) was dried for 24 hr *in vacuo* over P_2O_5 at 56°. Anhydrous hydrazine (10 ml) was added and the reaction mixture was maintained at 100° in a sealed tube for 8 hr

according to the procedure described by Tarcsay *et al.* (1971). Hydrazine was removed *in vacuo* over concentrated sulfuric acid and the residue, dissolved in 20 ml of water, was extracted three times with chloroform. The aqueous phase was adjusted to pH 7.0 by the addition of 1 N HCl, concentrated to small volume (3 to 5 ml), and applied onto a Sephadex G-50 column (2.5 × 100 cm). The column was eluted with distilled water. A rather broad elution pattern of the resulting, deacylated O-specific side chain indicated some degradation of the polysaccharide chain by hydrazine treatment (Yosizawa and Sato, 1962). Only the large molecular weight fraction corresponding to the first 100 ml of eluate was collected. After lyophilization, approximately 200 mg of the deacylated polysaccharide was obtained.

N-Acetylation of D-*Glucosamine.* Oligosaccharide fragments containing D-glucosamine were N-acetylated according to the procedure of Roseman and Ludowieg (1954). The aqueous solution (1 ml) of oligosaccharide containing 0.5–5.0 mg of D-glucosamine was mixed with 1.5 ml of a 0.5% solution of $NaHCO_3$ and 150 μl of acetic anhydride at 4°. The mixture was kept at room temperature for 24 hr. The reaction mixture was passed through a mixed-bed ion-exchange column and lyophilized.

Preparation of Methyl Glycoside of the D-*Glucosyl*-D-*Galactose Disaccharide and Its Periodate Oxidation.* Disaccharide (5 mg) was stirred with 3 ml of absolute methanol and 0.5 g of Dowex 50-H^+ resin (dried over P_2O_5 before use) for 3 hr under reflux. After cooling, the reaction mixture was centrifuged and the resin was washed three times with methanol. After methanol had been removed by vacuum distillation, the reaction product was purified by paper chromatography with solvent system A (*n*-butyl alcohol–pyridine–water, 6:4:3, v/v). The disaccharide methyl glycoside was oxidized with 0.05 M sodium periodate (2 ml) for 24 hr in the dark at room temperature. The excess periodate was destroyed by the addition of 50 μl of ethylene glycol, and the remaining reaction mixture was treated with sodium borohydride (20 mg) for 16 hr at 4°. The solution was acidified with acetic acid and the borate was removed by repeated vacuum evaporation of the reaction mixture with methanol. The residue was desalted by high-voltage paper electrophoresis; the neutral band was eluted with distilled water and hydrolyzed with 1 N HCl for 4 hr at 100°. The hydrolysate was evaporated to dryness over NaOH in a vacuum desiccator and the residue was acetylated and subjected to gas–liquid chromatography.

Paper Chromatography, High-Voltage Paper Electrophoresis, and Agar Gel Electrophoresis. Paper chromatography was carried out with the following solvents: A, butyl alcohol–pyridine–water (6:4:3, v/v); B, ethyl acetate–pyridine–water (360:100:115, v/v, organic phase).

High-voltage paper electrophoresis was performed in a Savant electrophoresis tank, Model LT-48A, with a power supply, Model HV-5000-3TC (Savant Instruments, Inc., Hicksville, N. Y.). A buffer of pyridine–acetic acid–water (10:4:86, v/v, pH 5.3) was used at 2500 V for 30–40 min.

Descending paper chromatography and electrophoresis were carried out on Whatman No. 1 or Schleicher & Schüll No. 2043b papers. Reducing sugars were detected with alkaline silver nitrate (Trevelyan *et al.*, 1950). Sugar glycosides were detected by spraying the papers with sodium periodate solution and then treating them with alkaline silver nitrate (Osborn, 1963).

Agar electrophoresis was carried out in 1% agar according to the procedure of Grabar and Williams (1955), employing barbital buffer, pH 8.6, ionic strength 0.05. The O-specific side chain was dissolved in the same buffer (20 mg/ml) and diluted to a final concentration of 10 mg/ml with 1% agar. Electrophoresis was performed for 40 min. After the plate had been fixed in 95% ethanol for 3 hr, the polysaccharide was detected as a precipitation band. The polysaccharide band was also detected by staining with periodic acid–Schiff reagent, as described for the polyacrylamide gel (Zacharius *et al.*, 1969). However, periodate oxidation was carried out for 7 rather than 50 min. The polysaccharide appeared as a dark red band on a light red background.

Gas–Liquid Chromatography and Mass Spectrometry. Gas–liquid chromatography of acetylated sugars was performed with a Barber-Colman gas chromatograph, Series 5000, equipped with a flame detector. The glass column was packed with 3% (w/w) ECNSS-M on Gas Chromosorb Q, 80–100 mesh. The combined gas–liquid chromatography and mass spectrometry was performed with a Perkin-Elmer Model 270B mass spectrometer according to the precedure of Hellerqvist *et al.* (1968).

Analytical Ultracentrifugation. Ultracentrifugal analyses were carried out in a Spinco Model E ultracentrifuge equipped with a phase schlieren diaphragm and interference optics, and an automatic temperature-control unit. Sedimentation rate was determined at constant temperature (25–26°) using a rotor speed of 56,100 rpm. The sedimentation coefficient was calculated as described by Schachman (1957). The molecular weight of the O-specific side chain was estimated by the sedimentation equilibrium procedure of Yphantis (1960), using a rotor speed of 33,450 rpm. The O-specific side chain was dissolved in 0.15 M NaCl.

The partial specific volume of the O-specific side chain was estimated by a pycnometric method (Kabat and Mayer, 1961). The value reported is the average from three determinations.

Infrared and Nuclear Magnetic Resonance (nmr) Spectroscopy. Infrared spectra were recorded with a Beckman infrared spectrophotometer IR10 using potassium bromide pellets (0.5 mg of substance and 250 mg of KBr). The nmr spectroscopy was performed by Sadtler Research Laboratory, Inc., Philadelphia, Pa. The samples were examined in a Varian A-60A proton magnetic spectrometer. The determinations were made at a probe temperature of 36°.

Immunological Methods. The immunological properties of the O-specific side chain were studied by double diffusion (Ouchterlony, 1953) and immunoelectrophoresis (Grabar and Williams, 1955) in 1% agar gel using barbital buffer, pH 8.6, ionic strength 0.1. Plates allowed to develop for 24–30 hr were washed several times with 0.15 M NaCl and distilled water and dried at room temperature. They were stained with Amido Black 10B.

For the preparation of antisera, intact endotoxin was used as antigen. White rabbits were immunized by successive intraperitoneal injections of 0.5 mg of the antigen suspended in 1 ml of 0.9% saline solution and 1 ml of Fruend's complete adjuvant at weekly intervals (total, four injections). Animals were bled by cardiac puncture at the end of the fourth week. The presence of antibodies was tested by double diffusion technique in agar gel.

Hemagglutination Inhibition Test. Twenty-five microliters of a 3% solution of oligosaccharides in 0.155 M phosphate buffer, pH 7.4, was added to 25 μl of a 1:64 titer anti-B serum (Ortho Diagnostics, Raritan, N. J.). After incubation for 30 min at room temperature 25 μl of a 1% solution of 0.155 M phosphate buffer, pH 7.4, containing type B red blood cells, was added and the plates were incubated for another 30 min. Blood-group B substance (Pfizer Laboratories, New York,

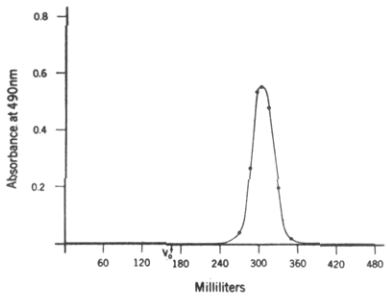


FIGURE 1: Gel filtration chromatography of the O-specific side chain on Sepharose 4B. The column (2.5 × 100 cm) was eluted with 0.15 M NaCl in 0.05 M phosphate buffer (pH 7.0), and 3-ml fractions were collected. The flow rate was 16 ml/hr. The column was monitored by determining carbohydrates with the phenol–sulfuric acid method.

N. Y.) served as the positive control, while phosphate buffer and a 3% lactose solution in phosphate buffer served as the negative controls.

Results

Characterization of the O-Specific Side Chain. The O-specific side chain was eluted from the Sepharose 4B column as a sharp symmetrical peak (Figure 1). It moved as a single band toward the cathode during agar gel electrophoresis (Figure 2) and gave a single immunoprecipitin line with antibodies to intact endotoxin (Figure 3). It was also characterized by a single, symmetrical boundary in the analytical ultracentrifuge (Figure 4). The absence of amino acids, fatty acids, heptose, and 2-keto-3-deoxyoctulonic acid indicated that the purified O-specific side chain was free of contamination by oligosaccharide core and conjugated protein. Elemental analysis indicated the absence of phosphorus. High-voltage paper electrophoresis disclosed no migrating spots staining with ninhydrin or alkaline silver nitrate solutions. The infrared spectrum (Figure 5) was characterized by strong absorption bands at 3400–3280 ($\bar{\nu}_{OH}$ associated) and 1060 cm^{-1} ($\bar{\nu}_{C-O}$ and $\bar{\gamma}_{OH}$ carbohydrate), and by the amide I and amide II at 1650 and 1520 cm^{-1}, respectively. The ester absorption band was absent. Gas–liquid chromatography and colorimetric sugar analyses indicated that D-glucose (20.2%), D-galactose (19.8%), and D-glucosamine (40.6%) were the only monosaccharides present, in a molar ratio of 1:1:2. The nmr spectrum (Figure 6) showed the presence of an acetyl group (τ 8.0). The absence of an ester absorption band and the presence of both amide absorption bands in the infrared spectrum of the O-specific side chain strongly suggested that the acetyl group was linked exclusively to the amino group of D-glucosamine. These results implied that the O-specific side chain probably consisted of a tetrasaccharide repeating unit.

The O-specific side chain was characterized by a sedimentation coefficient, $s_{20,w}$, of 1.20 S and a partial specific volume of 0.620 ml/g. The molecular weight, determined by the sedimentation equilibrium method, was 12,400.

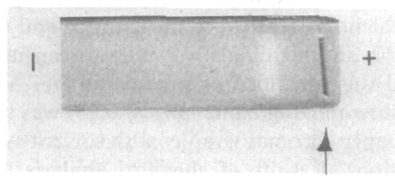


FIGURE 2: Agar gel electrophoresis of the O-specific side chain. A sample (20 mg/ml) was dissolved in barbital buffer, pH 8.6, ionic strength 0.05, and diluted to a final concentration of 10 mg/ml with 1% agar. Electrophoresis was performed in 1% agar gel using barbital buffer for 40 min. A photograph was taken after the plate had been fixed in 95% EtOH.

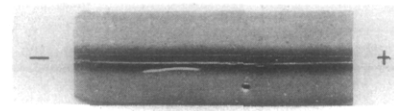


FIGURE 3: Immunoelectrophoretic pattern of the O-specific side chain. The polysaccharide was dissolved in barbital buffer (10 mg/ml), pH 8.6, ionic strength 0.1. The electrophoresis was carried out for 40 min. The central trough contains antibodies to intact endotoxin.

Determination of the Sugar Sequence in the Repeating Unit of the O-Specific Side Chain. Fragmentation of the O-specific side chain by treatment with periodate, sulfuric acid, or hydrazine, followed by isolation and characterization of the resulting oligosaccharide fragments, was used to reconstruct the monosaccharide sequence in the repeating unit of the side chain.

Periodate oxidation of the O-specific side chain resulted in the consumption of 3 mol of periodate/mol of tetrasaccharide. After sodium borohydride reduction and mild acid treatment (Smith degradation) of the oxidation product, the reaction mixture was passed through a Sephadex G-15 column. Monitoring of the eluates with phenol–sulfuric acid reagent revealed a single carbohydrate-positive peak characterized by an elution volume between that of D-raffinose (trisaccharide) and lactose (disaccharide). Quantitative analysis of this fragment, designated oligosaccharide 01, showed the presence of equimolar amounts of *N*-acetyl-D-glucosamine, D-galactose, and glycerol. Thus, periodate oxidation degraded all of the D-glucose and half of the initial *N*-acetyl-D-glucosamine. Periodate oxidation of oligosaccharide 01 caused the degradation of D-galactose. These results suggested that the sugar sequence of oligosaccharide 01 was D-Gal→D-*GlcNAc*→glycerol.

Additional oligosaccharide fragments were isolated by partial acid hydrolysis as follows. The O-specific side chain (100 mg) was subjected to acid hydrolysis by 0.5 N H_2SO_4 for

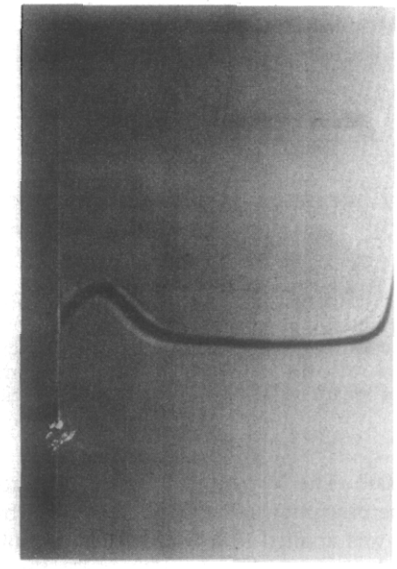


FIGURE 4: The ultracentrifugal pattern of the O-specific side chain. The sample (10 mg/ml) dissolved in 0.15 M NaCl was centrifuged at 52,640 rpm at 25°. Exposure was taken from left to right 40 min after reaching full rotor speed.

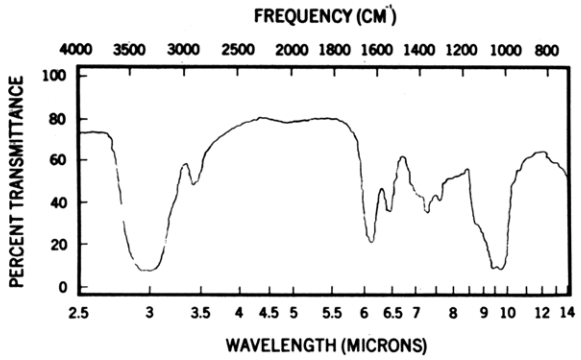


FIGURE 5: Infrared spectrum of O-specific side chain (0.5 mg/250 mg of KBr).

tively. Fraction II was further purified by preparative paper chromatography with solvent system A. A silver nitrate positive band with a mobility (R_{Glc} 0.30) resembling that of lactose was eluted from the paper; this material (11 mg) was designated as oligosaccharide 02. It migrated as a single spot when chromatographed with solvent system B. Quantitative sugar analysis showed equimolar amounts of D-glucose and D-galactose. After sodium borohydride reduction and 1 N hydrochloric acid hydrolysis of oligosaccharide 02, D-galactose could not be detected by the D-galactose oxidase method. On the other hand, the amount of D-glucose remained unchanged. These results indicated that the sugar sequence of oligosaccharide 02 was D-Glc→D-Gal.

Since glycopyranosides of 2-amino-2-deoxyhexoses are resistant to acid hydrolysis (Lloyd and Evans, 1968), the isolation of D-glucosamine-containing fragments was attempted by hydrazinolysis of the O-specific side chain. The O-specific side chain was deacetylated by treatment with hydrazine and the reaction mixture was applied onto a Sephadex G-50 column and eluted with distilled water. A broad elution pattern of the deacylated O-specific side chain indicated some degradation of polysaccharide chain by hydrazine treatment. Since the sole purpose of this reaction was to isolate a glucosamine containing oligosaccharide by further acid hydrolysis of the resulting hydrazinolysate, the isolation of a deacylated O-specific side chain of uniform size was not attempted. Arbitrarily, only fractions corresponding to the first 100-ml eluate were pooled and hydrolyzed with 1 N H_2SO_4 for 3 hr at 100°. After neutralization with $Ba(OH)_2$ and concentration, the reaction mixture was subjected to preparative high-voltage paper electrophoresis. The electropherogram showed two bands which moved toward the cathode and reacted with both ninhydrin and alkaline silver nitrate reagents. The fast-moving band was identified as D-glucosamine. After N-acetylation, the slow-moving band (M_{GlcN} 0.65) was separated by preparative paper chromatography with solvent system B into two subfractions. Results of chemical analysis showed that the fast-moving subfraction (R_{Glc} 0.60), designated oligosaccharide 03, contained equimolar amounts of *N*-acetyl-D-glucosamine and D-galactose, with the latter sugar at the reducing end. The slow-moving subfraction (R_{Glc} 0.51), called oligosaccharide 04, consisted of equimolar quantities of *N*-

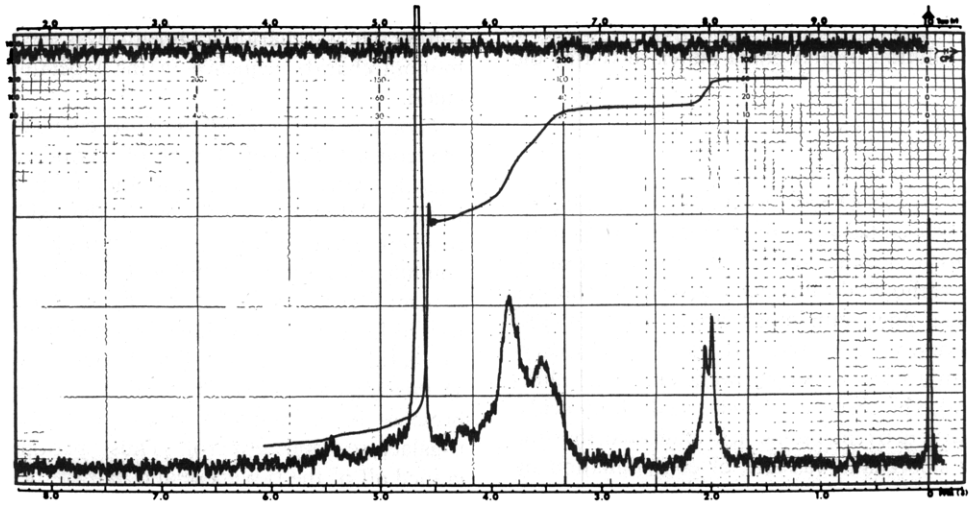


FIGURE 6: Nuclear magnetic resonance spectrum of the O-specific side chain dissolved in deuterium oxide.

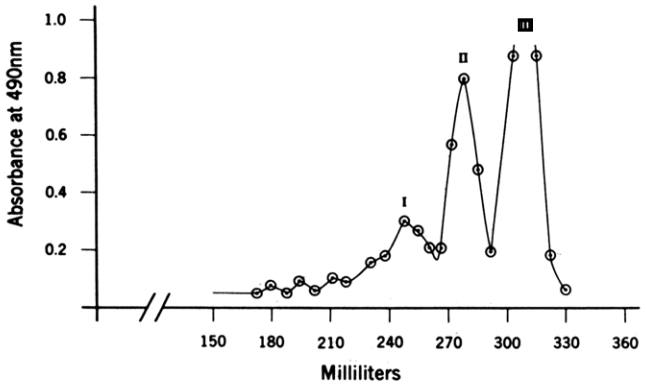


FIGURE 7: Gel filtration chromatography of a partially hydrolyzed O-specific side chain on Sephadex G-15. The column (2.5 × 100 cm) was eluted with distilled water, and 3-ml fractions were collected. The flow rate was 30 ml/hr. The column was monitored by determining carbohydrates with the phenol–sulfuric acid method.

70 min at 100°. The hydrolysate was neutralized with $Ba(OH)_2$ and the precipitated $BaSO_4$ was removed by filtration. After filtrate was applied to a Sephadex G-15 column, three distinct carbohydrate-positive fractions were eluted with distilled water (Figure 7). Peak I represented a trisaccharide or a longer oligosaccharide chain, whereas peaks II and III corresponded to a disaccharide and monosaccharide, respec-

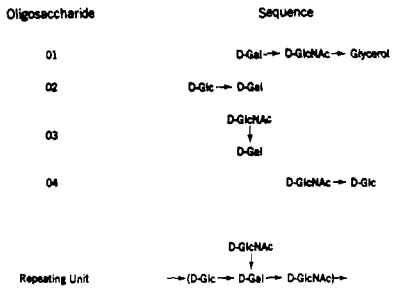


FIGURE 8: Reconstruction of the repeating unit of the O-specific side chain.

acetyl-D-glucosamine and D-glucose. The reducing end of this disaccharide fragment was determined to be D-glucose.

We concluded from the results of these experiments that the sugar sequence (Figure 8) of the repeating unit of the O-specific side chain is

D-GlcNAc
↓
→D-Glc→D-Gal→D-GlcNAc→

Determination of the Sugar Linkages in the Repeating Unit of the O-Specific Side Chain. To determine the linkages of D-glucose and D-galactose, the O-specific side chain was permethylated and hydrolyzed. The methylated monosaccharides were reduced and acetylated, and the derivative of methylated D-glucosamine was removed from the reaction mixture by ion-change chromatography on Dowex 50-H^+. Gas–liquid chromatography of the derivatives of methylated D-glucose and D-galactose disclosed three peaks (Figure 9). Peak I was identified as the alditol acetate of 2,3,4,6-tetra-*O*-methyl-D-glucose and peak II as that of 2,3,4-tri-*O*-methyl-D-glucose. This was done by comparing their relative retention times with those of authentic sugars. The identity of these two methylated D-glucose derivatives was verified by mass spectrometry. The mass spectrum of the methylated sugar in peak III was characterized by major fragments (>10% of the base peak, *m/e* 43, 45, 87, 117, and 129). These fragments are typical of the alditol acetates of 2,6-di-*O*-methylhexoses (Björndal *et al.*, 1970).

The identification of 2,3,4,6-tetra-*O*-methyl-D-glucose was interpreted as proof of the presence of D-glucose at the nonreducing end of the O-specific side chain. On the other hand, the presence of 2,3,4-tri-*O*-methyl-D-glucose clearly showed that within the repeating unit D-glucose was substituted at C-6. These findings not only explained the complete degradation of D-glucose by periodate oxidation but also demonstrated that in oligosaccharide 01 the glycerol could be derived only from D-glucose. Since the first two peaks were identified as derivatives of D-glucose, the third peak, already characterized as a 2,6-di-*O*-methylhexose, could stem only from D-galactose. We concluded, therefore, that D-galactose was substituted at C-3 and C-4.

To establish whether D-galactose was substituted by D-glucose at C-3 or C-4, the oligosaccharide 02 was treated with absolute methanol in the presence of dried Dowex 50-H^+. The resulting purified methyl *O*-D-glucosyl-D-galactoside (Figure 10) was oxidized with periodate, reduced with sodium borohydride, and hydrolyzed (as described in Methods). The hydrolysate was neutralized and acetylated. Since the acetylated alditol was identified by gas–liquid chromatography as D-threitol, we concluded that D-galactose was substituted by

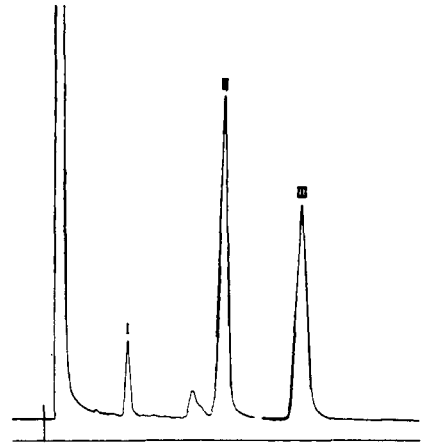


FIGURE 9: Gas–liquid chromatogram of the alditol acetates of methylated monosaccharides from the O-specific side chain.

D-glucose at C-4. From this experiment, we also deduced that in the repeating unit D-galactose was substituted by *N*-acetyl-D-glucosamine at C-3.

The only remaining linkage to be determined was that between D-galactose and *N*-acetyl-D-glucosamine in the linear part of the repeating unit. Since the branched *N*-acetyl-D-glucosamine would obviously be degraded by periodate oxidation of the O-specific sice chain, the nondegraded *N*-acetyl-D-glucosamine could have been substituted by D-galactose either at C-3 or C-4. To distinguish between these two alternatives, the O-specific side chain was treated with hydrazine and oxidized by periodate for 60 hr at room temperature. Quantitative sugar analysis showed that the reaction product contained equimolar amounts of D-glucosamine and D-galactose, much like the result found for periodate oxidation at the intact O-specific side chain. Thus, after deacetylation and oxidation of the O-specific side chain, the D-glucosamine of the linear portion of the chain was still preserved, indicating that D-galactose is glycosidically bonded to the C-3 of *N*-acetyl-D-glucosamine.

Anomeric Configuration of the O-Specific Side Chain. Nmr spectroscopy of the intact O-specific side chain in deuterium oxide (Figure 6) revealed the presence of both α (τ 4.6) and β (τ 5.0) anomeric proton signals (Whyte, 1971). Further studies on the anomeric configuration of constitutive sugars were performed with three available oligosaccharide fragments of known sequence (Figure 8). The positive hemag-

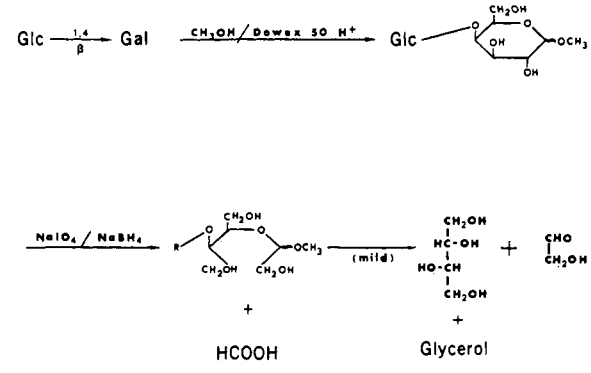


FIGURE 10: Preparation of methyl glycoside of disaccharide 02 and periodate oxidation of the methyl glucoside.

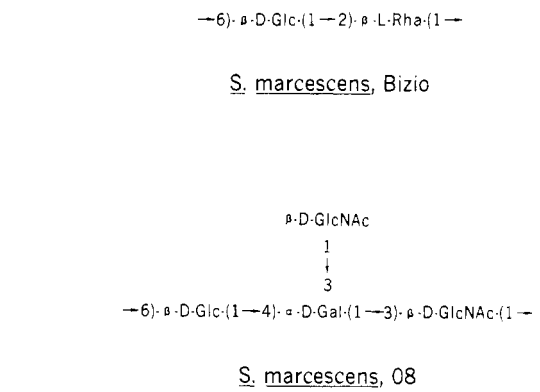
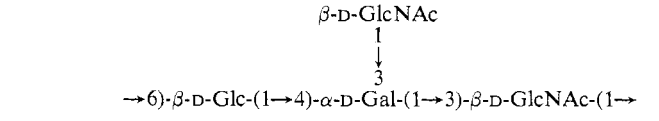


FIGURE 11: Structure and anomeric configurations of O-specific side chains from *S. marcescens*, Bizio, and *S. marcescens* 08.

glutination inhibition test of red blood cell B activity by oligosaccharide 01 indicated that D-galactose had an α configuration. This was confirmed by the release of 95% D-galactose with fig α-galactosidase. Combined treatment of oligosaccharide 01 with α-galactosidase and β-*N*-acetylhexosaminidase resulted in an almost quantitative (95–97%) cleavage of D-galactose and *N*-acetyl-D-glucosamine. On the other hand, the combined treatment of oligosaccharide 01 with β-galactosidase and β-*N*-acetylhexosaminidase failed to cleave any of the glycosidically linked sugars. This not only established the α-anomeric configuration of D-galactose and the β-anomeric configuration of *N*-acetyl-D-glucosamine, it also confirmed that the sugar sequence in oligosaccharide 01 is D-Gal→D-GlcNAc→glycerol.

The complete cleavage of D-glucose from disaccharide 02 by the action of β-glucosidase and that of *N*-acetyl-D-glucosamine from disaccharide 03 by β-*N*-acetylhexosaminidase clearly indicated that D-glucose and both *N*-acetyl-D-glucosamine residues in the repeating unit had β configurations.

Structure of the O-Specific Side Chain. From studies on the sequence, linkages, and anomeric configurations of the constitutive monosaccharides, we concluded that the O-specific side chain of the chromogenic strain *S. marcescens* 08 has the following structure.



Since the molecular weight determined by the sedimentation equilibrium method was 12,400, the O-specific side chain was calculated to contain, on the average, 17 repeating units.

Discussion

The isolation and identification of characteristic oligosaccharide fragments (Figure 8) after partial acid hydrolysis or Smith degradation provided conclusive evidence that the O-specific side chain of *S. marcescens* 08 consists of repeating units rather than randomly distributed monosaccharides. These findings represent further proof that the polysaccharide moieties of endotoxin preparations from the genus *Serratia* are composed of the same structural entities as those from *Salmonella*, *Escherichia*, and *Shigella* (Osborn, 1969). Most repeating units from these latter genera of Enterobacteriaceae contain a variety of linear trisaccharides with or without additional branched sugars. The repeating unit of the O-specific side chain of *S. marcescens* 08 shares this basic structural pattern with side chains of other Gram-negative organisms.

The permethylation studies and the resistance of the O-specific side chain to 1% acetic acid hydrolysis offer clear evidence that all sugars of the repeating unit occur in the pyranosidic form. Furanosyl linkages are highly acid labile (Haworth, 1932) and would be at least partially cleaved during the procedure used to isolate the O-specific side chain.

A striking compositional and structural difference emerged between the repeating units of a chromogenic and a nonchromogenic strain of *S. marcescens* (Figure 11). In contrast to the more common type of branched tetrasaccharide repeating unit found in the pigmented *S. marcescens* 08, the repeating unit of the nonpigmented *S. marcescens* Bizio is built of a simple D-glucose-L-rhamnose disaccharide (Wang and Alaupovic, 1973). Immunological studies showed that only the O-specific side chain from *S. marcescens* 08 gave a positive immunoprecipitin reaction with its homologous antiserum. Furthermore, no cross-reactivity was observed between the O-specific side chains of these two strains.

Intravenous injections of O-specific side chain from *S. marcescens* 08 in doses up to 100 mg/kg had no toxic effects in mice.

Acknowledgments

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Heterogeneity of Carbohydrate Fragments Isolated from Human Blood Group H and Le^a Active Glycoproteins by Base–Borohydride Degradation†

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ABSTRACT: Degradation of two blood group substances possessing H and Le^a activity by 0.05 N NaOH and 1.0 M $NaBH_4$ gave a mixture of oligosaccharide chains all ending with *N*-acetyl-D-galactosaminitol with minimal amounts of degradation products. Passage of the dialyzable material on Bio-Gel P-2 revealed substantial heterogeneity evident primarily from the presence of several peaks and from the distribution of fucose. In the H active substance this sugar was present in several included peaks while it was restricted almost exclusively to the excluded carbohydrate fraction of the Le^a active glycoprotein. Although the gel filtration profiles of the two dialysates were generally similar, both showing a large excluded portion containing the more complete megalosaccharide chains and several additional included peaks, the H oligosaccharides had more material of larger size. All peaks were heterogeneous on analytical paper and on charcoal chromatography, and contained incomplete oligosaccharide chains decreasing in size and composition down to *N*-acetyl-D-galactosaminitol. Evidence is presented that some of the nondialyzable fraction had also undergone alkaline β elimination from the protein with the formation of large reduced oligosaccharides terminated by *N*-acetyl-D-galactosaminitol.

The series of oligosaccharides isolated in this and in other laboratories from blood group active glycoproteins by various degradation procedures such as mild acid hydrolysis and alkaline or alkaline borohydride degradation have been fundamental for establishing the structure of the blood group specific determinants and for the elucidation of the general structure of blood group active glycoproteins (Painter and Morgan, 1961; Schiffman *et al.*, 1964; Marr *et al.*, 1967; *cf.* Kabat, 1970, 1973, for reviews). It has also become possible to assemble them into a proposed overall composite structure (Lloyd *et al.*, 1968; Lloyd and Kabat, 1968; Vicari and Kabat, 1970; Lundblad *et al.*, 1972), accounting for almost all of the carbohydrate chains isolated from blood group A, B, H, Le^a, and from a precursor substance, shown to possess blood group I activity (Feizi *et al.*, 1971a,b) as well as for the products of alkaline degradation. This composite structure is thought to represent a relatively complete type of carbohydrate chain. Since experimental data always indicated that the carbohydrate moiety of blood group active glycoproteins showed substantial heterogeneity, individual chains could occur in the proposed more complete megalosaccharide, or in various stages of completion all the way down to single *N*-

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