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

C. S. Wang and P. Alaupovic*

ABSTRACT: The endotoxin complex of *Serratia marcescens* Bizio was hydrolyzed by 1% acetic acid, and the O-specific side chain was isolated from the hydrolysate by dialysis and gel filtration on Sephadex G-100. The determinations of chemical composition and molecular weight indicated that the purified O-specific side chain was a polysaccharide consisting of equimolar quantities of D-glucose and L-rhamnose. On the basis of the evidence obtained from periodate oxidation, methylation, infrared spectroscopy, and partial acid hydrolysis, it was concluded that the O-specific side chain is a linear polysaccharide consisting of repeating units of a D-glucose–L-rhamnose disaccharide. The structure of the repeating unit was identified as →6)-β-D-Glc-(1→2)-β-L-Rha-(1→. Enzymatic hydrolysis of isolated disaccharide fractions with glucosidases and hesperidinase and nuclear magnetic resonance spectroscopy of the O-specific side chain indicated that both D-glucose and L-rhamnose have the β-anomeric configuration. The average number of repeating units in the O-specific side chain was estimated to be 43. This report presents the first structural elucidation of an O-specific side chain from genus *Serratia*. It also indicates that the O-specific side chains of Gram-negative bacteria may be composed of simple disaccharide repeating units.

Results of our recent studies (Wober and Alaupovic, 1971; Wang, 1971) have indicated that endotoxin preparations isolated by trichloroacetic acid extraction of *Serratia marcescens* 08 and *S. marcescens* Bizio consist of covalently linked polysaccharide, lipid, and protein moieties. Two fragments designated as conjugated protein and "degraded polysaccharide" were isolated from the acetic acid hydrolysates of these endotoxin preparations. Conjugated protein was characterized as an endotoxic fragment composed of intact protein and lipid moieties. The degraded polysaccharide was further fractionated by dialysis or Sephadex gel filtration (Müller-Seitz *et al.*, 1968; Fensom and Meadow, 1970; Romanowska and Lachowicz, 1970) into two fractions corresponding to the O-specific side chain and core fragments of the polysaccharide moiety. It is generally accepted (Lüderitz *et al.*, 1968; Osborn, 1969) that the macromolecular O-specific side chains are composed of a wide variety of oligosaccharide repeating units responsible for the serological specificity of each bacterial species. On the other hand, it seems that the single-unit oligosaccharide cores are limited to only a few, if not a single, compositional and structural entities characteristic of each bacterial genus (Lüderitz, 1970; Schmidt *et al.*, 1970). Isolation of these two polysaccharide fragments suggested strongly that the basic structural features of the polysaccharide moiety of endotoxins from *S. marcescens* may be similar to those from *Salmonella* (Lüderitz, 1970), *Escherichia* (Heath *et al.*, 1966), and *Shigella* (Simmons, 1969). However, in contrast to the successful elucidation of the detailed structure of the O-specific side chains and cores from some of these latter genera, commensurate information regarding the structure of polysaccharide moieties from various strains of *S. marcescens* is not available.

The results of our studies on the composition and structure of intact endotoxins from a chromogenic and a nonchromogenic strain of *S. marcescens* have indicated that the polysaccharide moieties from both bacterial strains contain a macromolecular side chain composed of repeating oligosaccharide units and a separate oligosaccharide core. In this paper, we describe the isolation and structure of the O-specific side chain from the nonchromogenic strain *S. marcescens* Bizio. Results show that this O-specific side chain consists of a unique disaccharide repeating unit of following structure: →6)-β-D-Glc-(1→2)-β-L-Rha(1→.

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Materials and Methods

Authentic Sugars. The 2,3,4-tri-*O*-methyl-D-glucose was obtained from D-raffinose (K & K Laboratories, Inc., Plainview, N. Y.). Permethylation and acid hydrolysis of D-raffinose (Courtois and Wickström, 1950) resulted in the formation of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose, and 1,3,4,6-tetra-*O*-methyl-D-fructose. After reduction and acetylation the alditol acetates of the methylated sugars were separated by gas–liquid chromatography and characterized by their relative retention times. As expected, the 2,3,4-tri-*O*-methyl-D-glucose exhibited the longest retention time; the structure of this methylated alditol acetate was confirmed by gas–liquid chromatography and mass spectrometry (performed in the Max-Planck Institute for Immunobiology, Freiburg, Germany, through the courtesy of Dr. H. Mayer).

A sample of 3,4-di-*O*-methyl-L-rhamnose was a gift from Dr. Bhavanandan. The preparation and characterization of 3,4-di-*O*-methyl-L-rhamnose were described by Aspinall and Bhavanandan (1965).

Bacteria. Several batches of cells of the nonchromogenic strain *S. marcescens* Bizio (American Type Culture Collection; strain 264), cultivated as described previously (Alaupovic *et al.*, 1966), were supplied by General Biochemicals, Chagrin Falls, Ohio. The bacterial cells were harvested in late logarithmic phase by centrifugation in a Sharples continuous-flow centrifuge and washed with distilled water.

Isolation of the O-Specific Side Chain. Wet cells were extracted twice with 5% trichloroacetic acid (2 l. of trichloroacetic acid/kg of cells) according to a modified method of Boivin *et al.* (1933). The combined extracts were dialyzed first against running tap water for 48 hr and then against distilled water for 24 hr, concentrated *in vacuo* to a small volume (approximately 300 ml), and centrifuged in a Spinco Model L ultracentrifuge at 105,000*g* for 1 hr. To remove free lipids the lyophilized nucleic acid free sediment (whole endotoxin) was extracted by chloroform–methanol (2:1, v/v) in a Soxhlet extractor for 24 hr. The residue represented the purified whole endotoxin (0.5–1% yield calculated on the basis of wet cells).

Purified whole endotoxin (1 g) was dissolved in 500 ml of preheated 1% acetic acid and hydrolyzed for 4 hr with stirring at 90° according to the procedure by Morgan and Partridge (1941). After cooling, the precipitate was separated from the clear supernatant by centrifugation at 27,000*g* for 30 min at 10°. The supernatant was extracted three times with chloroform. The lyophilized aqueous phase was dissolved in 100 ml of distilled water and centrifuged at 105,000*g* for 3 hr to remove the residual undegraded whole endotoxin. After repeating the entire step, the supernatant was lyophilized and the resulting yellow substance designated as degraded polysaccharide was dialyzed against distilled water for 7 days. The inner dialysate contained the *O-specific side chain*, while the outer dialysate contained mainly the *polysaccharide core*. The O-specific side chain was further purified by gel filtration on Sephadex G-100. Approximately 200 mg of the lyophilized inner dialysate was dissolved in 3 ml of distilled water and applied onto a column (100 × 2.5 cm). The column was eluted with distilled water at a flow rate of 30 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 anthrone method (Koehler, 1952). The combined and lyophilized anthrone-positive fractions represented the purified O-specific side chain. The elution volume of O-specific side chain was 230 ml. The void volume determined by Blue Dextran was 114 ml.

Periodate Oxidation of O-Specific Side Chain. The consumption of periodate was followed by measuring the decrease in absorption of periodate ion at 222.5 nm according to the procedure by Aspinall and Ferrier (1957).

Methylation Analysis of O-Specific Side Chain. The O-specific side chain was methylated by Hakomori's method as described by Sanford and Conrad (1966). The methylated polysaccharide was hydrolyzed for 4 hr with 1 N HCl in aqueous 90% methanol at 100°. The resulting methylated monosaccharides were reduced, acetylated, and analyzed by gas chromatography employing a glass column packed with 3% ECNSS-M on gaschrom Q, 80–100 mesh (Holme *et al.*, 1968). Xylitol pentaacetate was used as an internal standard.

Partial Acid Hydrolysis of the O-Specific Side Chain. The O-specific side chain (200 mg) was hydrolyzed with 0.5 M HCl for 2 hr at 100°. The hydrolysate was dried *in vacuo* by rotatory evaporation. The residue was dissolved in 3 ml of distilled water and chromatographed on a Sephadex G-15 column (100 × 2.5 cm) with distilled water as eluting solvent. The combined disaccharide fractions were purified by preparative paper chromatography employing solvent system a.

Enzymatic Hydrolysis of Disaccharides. HYDROLYSIS BY α-GLUCOSIDASE. α-Glucosidase (0.3 ml; Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml in water) was added to disaccharide solution (0.3 ml; 1 mg/ml) and 0.5 ml of 0.1 M phosphate buffer, pH 7.0. After incubation for 12 hr at 37°, 0.5 ml of the reaction mixture was assayed for free glucose by the glucose oxidase method.

HYDROLYSIS BY β-GLUCOSIDASE. Disaccharide solution (0.2 ml; 1 mg/ml) was incubated with 0.45 ml of a β-glucosidase solution (Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml in 0.1 M sodium acetate buffer, pH 5.0) for 6 hr at 37°. The release of glucose was determined by the glucose oxidase method.

HYDROLYSIS BY HESPERIDINASE. A hesperidinase solution (0.5 ml; Miles Laboratory, Inc., Elkhardt, Ind.; 1 mg/ml in 0.1 M sodium acetate buffer, pH 3.5) was incubated with 0.5 ml of disaccharide solution (1 mg/ml) for 12 hr at 37°. After incubation, the reaction mixture was desalted by ion-exchange column (5 × 0.5 cm) chromatography on Dowex 50W-X8 (H^+) and the released monosaccharide was identified by paper chromatography. When the substrate was L-rhamnosyl-D-glucose, the assay was also based on the release of D-glucose determined by the glucose oxidase method.

Analytical Methods. ANTHRONE-POSITIVE CARBOHYDRATES were determined according to the method of Koehler (1952). REDUCING SUGARS were estimated by the method of Sömögyi and Nelson as described by Marais *et al.* (1966). Polysaccharide samples were hydrolyzed with 1 N HCl in sealed tubes for 4 hr at 100°. NEUTRAL SUGARS were also analyzed by gas chromatography of their reduced and acetylated derivatives (Holme *et al.*, 1968). D-GLUCOSE was determined by the glucose oxidase method (Hugget and Nixon, 1957) and D-GALACTOSE was analyzed by the galactose oxidase method (Avigad *et al.*, 1962) utilizing the standard Glucostat and Galactostat reagents (Worthington Biochem. Corp., Freehold, N. J.), respectively. HEXOSAMINES were determined by the method of Rondle and Morgan (1955). For this analysis, the sugar samples were hydrolyzed with 4 N HCl in sealed tubes for 6 hr at 100°. 2-KETO-3-DEOXYOCTULONIC ACID was estimated by the thiobarbituric acid method (Weissbach and Hurwitz, 1959). L-RHAMNOSE was determined according to the procedure by Dische and Shettles (1951). AMINO ACID AND FATTY ACID ANALYSES were performed as described previously (Wober and Alaupovic, 1971).

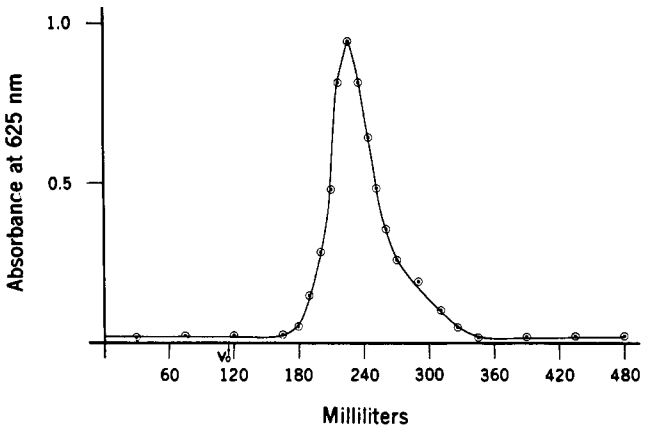

FIGURE 1: Gel filtration chromatography of O-specific side chain on Sephadex G-100. Experimental conditions: Column (2.5 × 100 cm) was eluted with distilled water. Volume of collected fractions was 3 ml/tube. Flow rate was 30 ml/hr. Column was monitored by determining the carbohydrate content with the anthrone method. The void volume was 114 ml.

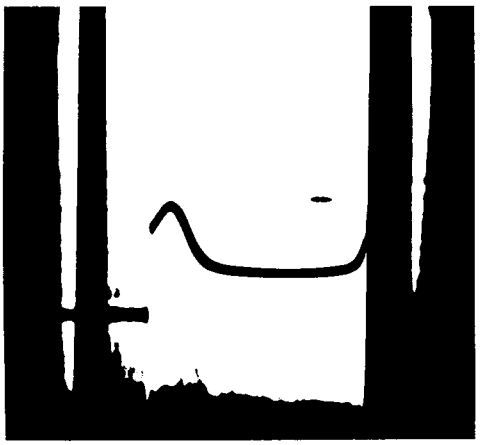

FIGURE 2: The ultracentrifugal pattern of the O-specific side chain. Sample (10 mg/ml) dissolved in 0.15 M NaCl was centrifuged at 52,640 rpm at 25°. Exposure was taken from left to right 40 min after reaching the speed.

Determination of Formaldehyde and Acetaldehyde. Aqueous solutions containing aldehydes were analyzed by gas–solid chromatography. Aliquots of reaction mixtures after periodate oxidation were injected directly into a glass column packed with Porapak QS, 80–100 mesh (Waters Association, Inc., Framingham, Mass.). The column temperature was 150°, while the injector and detector temperatures were 200 and 235°, respectively. The relative retention time of formaldehyde was 6.2 min and that of acetaldehyde was 7.2 min.

Paper Chromatography and High-Voltage Paper Electrophoresis. Separation of carbohydrates was carried out by descending chromatography on Whatman No. 1 paper. The solvent systems used were (a) ethyl acetate–pyridine–water (3.6:1:1.15, v/v) and (b) 1-butanol–acetic acid–water (4:1:5, v/v). High-voltage paper electrophoresis was performed at 3000 V for 30 min in a buffer system composed of pyridine–acetic acid–water (1:10:89, v/v), pH 3.65. Neutral sugars were detected by spraying the papers with alkaline silver nitrate (Trevelyan *et al.*, 1950) and amino sugars were detected with ninhydrin.

Analytical Ultracentrifugation. Ultracentrifugal analyses were carried out in a Spinco Model E ultracentrifuge equipped with a phase schlieren diaphragm, interference optics and an automatic temperature control unit. Sedimentation rate was determined at constant temperature (25–26°) with a rotor speed of 56,100 rpm. Sedimentation coefficient was calculated as described by Schachman (1957). The molecular weight of O-specific side chain was estimated by the sedimentation–equilibrium procedure of Yphantis (1960) with a rotor speed of 33,450 rpm. The O-specific side chain (0.5 mg/ml) was dissolved in 0.15 M NaCl.

The partial specific volume of O-specific side chain was estimated by a pycnometric method (Kabat and Mayer, 1961). The values obtained from three separate determinations were averaged.

Infrared and Nuclear Magnetic Resonance 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 nuclear magnetic resonance (nmr) spectroscopy was performed by Sadtler Research Laboratory, Inc., Philadelphia, Pa. The samples were examined on a Varian A-60A proton magnetic resonance spectrometer. Hydrogen–deuterium oxide was used as a solvent and internal standard. The determinations were made at a probe temperature of 36°.

Results

Isolation and Characterization of O-Specific Side Chain. The O-specific side chain separated from the polysaccharide core by exhaustive dialysis of degraded polysaccharide was further purified by Sephadex G-100 gel filtration. Eluted from the Sephadex G-100 column as a single sharp peak (Figure 1), the O-specific side chain was also characterized by a single, symmetrical boundary in the analytical ultracentrifuge (Figure 2). The absence of D-glucosamine, amino acids, fatty acids, heptose, and 2-keto-3-deoxyoctulonic acid showed that the purified O-specific side chain was free of contamination by polysaccharide core and conjugated protein. Elemental analysis indicated the absence of phosphorus and nitrogen. High-voltage electrophoresis disclosed no migrating spots staining with ninhydrin or alkaline silver nitrate solutions. The infrared spectrum (Figure 3) was characterized by strong absorption bands at 3400–3280 cm^{-1} ($\tilde{\nu}$ OH ass.) and 1060 cm^{-1} ($\tilde{\nu}$ C–O and γ OH carbohydrate); both amide and ester absorption bands were absent. The anthrone test showed that the O-specific side chain contained 97% carbohydrate using D-

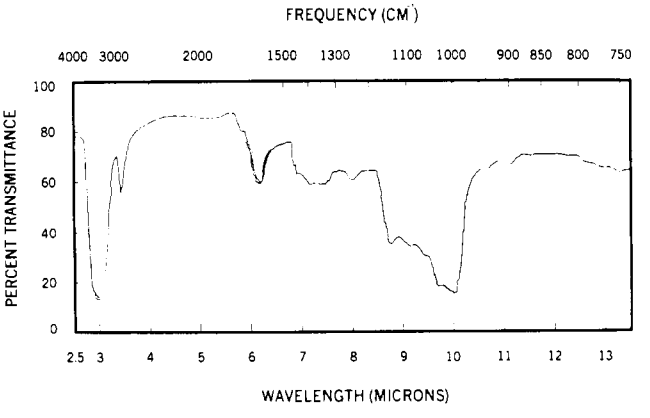

FIGURE 3: Infrared spectrum of O-specific side chain (0.5 mg/250 mg KBr).

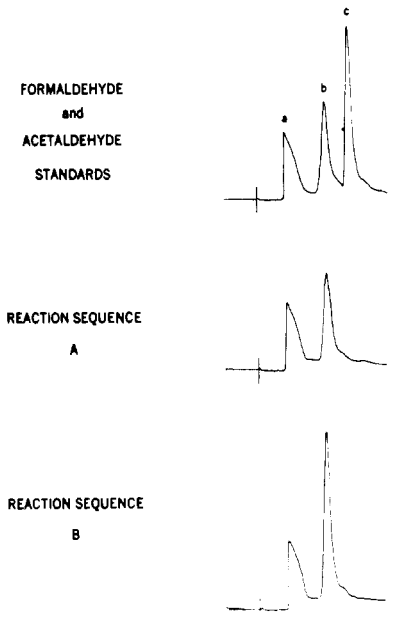

FIGURE 4: Gas–solid chromatography of formaldehyde and acetaldehyde formed by periodate oxidation of the methylated monosaccharides from O-specific side chain. Peak a H_2O, peak b formaldehyde, and peak c acetaldehyde.

glucose as a standard. The results of gas–liquid chromatography and colorimetric sugar analysis indicated D-glucose (55%) and L-rhamnose (55%) as the only monosaccharides present. The equimolar ratio (1:1.1) of D-glucose to L-rhamnose suggested strongly that the O-specific side chain consisted of a D-glucose–L-rhamnose disaccharide repeating unit.

The side chain was characterized by a sedimentation coefficient, $s_{20,w}$, of 1.77 S and a partial specific volume of 0.619 ml/g. The molecular weight, determined by the sedimentation equilibrium method, was 13,200 and that estimated from the reducing end analysis was 11,300. These results indicated that the O-specific side chain is a macromolecular compound with a single reducing end.

TABLE I: Theoretical Formation of Formaldehyde and Acetaldehyde from Methylated D-Glucose and L-Rhamnose.

L-Rhamnose Linkages	D-Glucose[a]		L-Rhamnose	
	Formaldehyde (mol)	Acetaldehyde (mol)	Formaldehyde (mol)	Acetaldehyde (mol)
Reaction sequence A[b]				
1→2	1	0	0	0
1→4	1	0	0	1
Reaction sequence B[c]				
1→2	1	0	1	0
1→4	1	0	0	1

[a] It is assumed that D-glucose is substituted at C-6. [b] Hydrolysate of permethylated O-specific side chain. [c] Hydrolysate of permethylated O-specific side chain subjected to borohydride reduction prior to periodate oxidation.

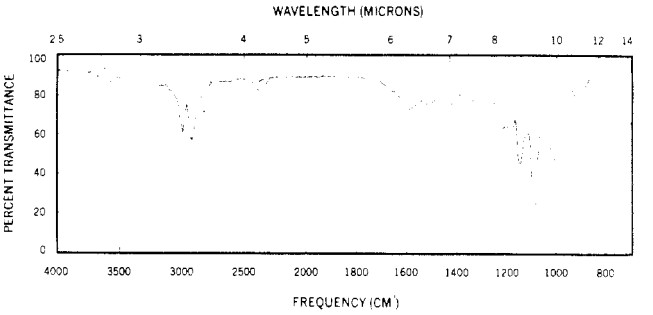

FIGURE 5: Infrared spectrum of methylated O-specific side chain in chloroform.

Determination of the Linkage of D-*Glucose and* L-*Rhamnose.* The complete degradation of the O-specific side chain by periodate oxidation (established by the failure to detect D-glucose or L-rhamnose in the reaction mixture by either paper chromatography or gas–liquid chromatography) indicated absence of 1→3 linkages. On the basis of an experimental periodate consumption of 2.8 mol/mol of D-glucose–L-rhamnose disaccharide, we have concluded that D-glucose must be substituted at C-6. The L-rhamnose, however, can be substituted either at C-2 or at C-4. To distinguish between these two alternative linkages, the O-specific side chain was subjected to the following sequence of reactions. The O-specific side chain was first reduced with sodium borohydride and the reduced product was methylated, dialyzed, and hydrolyzed with 1 N HCl. One aliquot of this reaction mixture was oxidized with periodate (reaction sequence A), while the other aliquot was first reduced with sodium borohydride and then oxidized with periodate (reaction sequence B). The theoretical formation of aldehydes from the methylated D-glucose and L-rhamnose is presented in Table I. It has already been established from the results of periodate oxidation that L-rhamnose is linked to C-6 of D-glucose. Therefore, 1 mol of formaldehyde should be generated from methylated D-glucose during both reaction sequences. If methylated L-rhamnose were substituted at C-2 in the native side chain, reaction sequence A would yield from each repeating unit 1 mol of formaldehyde and the reaction sequence B would result in the formation of 2 mol of formaldehyde. Neither reaction sequence would liberate acetaldehyde. If, on the other hand, methylated L-rhamnose were substituted at C-4 in the native side chain, both reaction sequences would generate 1 mol of acetaldehyde and 1 mol of formaldehyde. The results of gas–solid chromatography (Figure 4) of the reaction products showed that the formaldehyde ratio of reaction sequences B:A was 1.6:1 and that no acetaldehyde was formed. The absence of OH absorption in the infrared spectrum of the methylated O-specific side chain (Figure 5) confirmed the completeness of the methylation reaction and, therefore, the validity of the formaldehyde ratio of reaction sequences A and B. We have concluded from these findings that D-glucose is bound to L-rhamnose through a 1→2 linkage.

To confirm the results of periodate oxidation, methylated O-specific side chain was hydrolyzed with 1 N HCl, reduced with sodium borohydride and acetylated. The methylated alditol acetates were analyzed by gas–liquid chromatography. Identification of 3,4-di-*O*-methyl-1,2,5-tri-*O*-acetylrhamnitol (retention time 3.5 min) and of 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetylglucitol (retention time 7.0 min) was done by comparison with authentic samples. These findings confirmed that D-

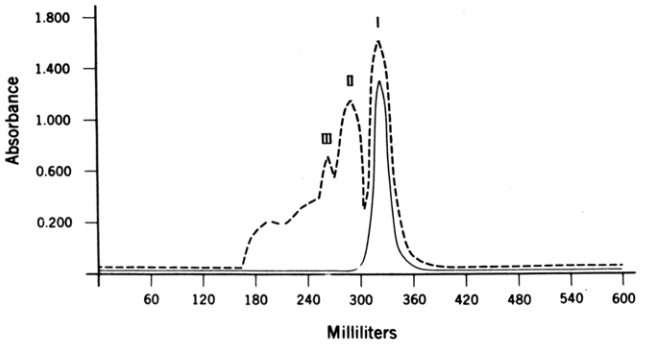

FIGURE 6: The gel filtration chromatography of partially hydrolyzed O-specific side chain on Sephadex G-15. Experimental conditions: column (2.5 × 100 cm) was eluted with distilled water. Volume of collected fractions was 3 ml/tube. Flow rate was 30 ml/hr. Column was monitored by determining the mixture of sugars with anthrone method (broken line) and glucose by glucose oxidase (solid line).

glucose was substituted at position 6 and L-rhamnose at position 2. The absence of di-*O*-methyl-D-glucose and mono-*O*-methyl-L-rhamnose derivatives indicated a linear rather than a branching structure of the polysaccharide.

Partial Hydrolysis of the O-Specific Side Chain. To establish that the O-specific side chain consists of repeating units of a D-glucose–L-rhamnose disaccharide, the polysaccharide was hydrolyzed with 0.5 N HCl for 2 hr at 100° and the hydrolysate was fractionated by gel filtration on Sephadex G-15. The elution pattern is shown in Figure 6. Fractions I, II, and III contained monosaccharides, disaccharides, and trisaccharides, respectively. Fraction II corresponding in its elution pattern to a disaccharide was separated by preparative paper chromatography into only two silver nitrate positive bands with mobilities between those of D-glucose and lactose (Figure 7). These two bands were designated D-1 (R_{glc} 0.66) and D-2 (R_{glc} 0.42), respectively.

Subfractions D-1 and D-2 were eluted and subjected to enzymatic degradation with α- and β-glucosidases and with

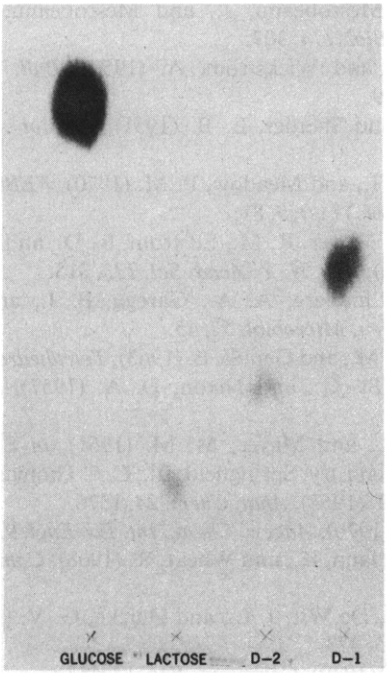

FIGURE 7: Paper chromatographic pattern of fractions D-1 and D-2 in solvent system a.

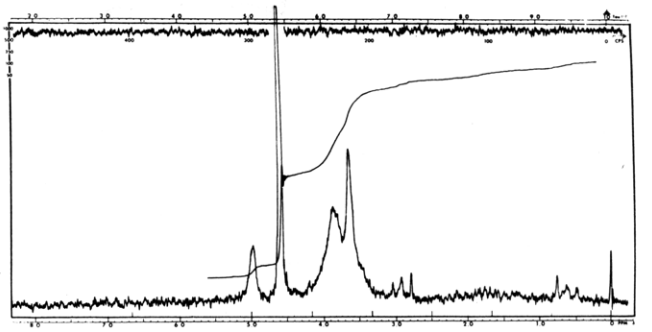

FIGURE 8: Nmr spectrum of O-specific side chain dissolved in hydrogen–deuterium oxide.

hesperidinase. Whereas α-glucosidase cleaves the nonreducing end of α-D-glucosides and β-glucosidase that of β-D-glucosides, the hesperidinase cleaves a corresponding bond of β-L-rhamnosides (Okada *et al*,, 1963a). The release of monosaccharides was assayed both by glucose oxidase method and by paper chromatography. The α-glucosidase had no effect on either D-1 or D-2 subfractions, while β-glucosidase catalyzed the hydrolysis of D-1 but not of D-2. Hesperidinase, on the other hand, cleaved only the D-2 subfraction. Since both subfractions consisted of equimolar quantities of D-glucose and L-rhamnose, it was concluded that the structure of D-1 is β-D-Glc-(1→2)-L-Rha and that of D-2 is β-L-Rha-(1→6)-D-Glc.

The partial hydrolysate of O-specific side chain also contained slow-moving reducing bands which correspond most probably to tri- and tetrasaccharides. These subfractions contained both D-glucose and L-rhamnose, but they were present in amounts too small for structural studies. However, results of the characterization of fraction II showed clearly the absence of either D-Glc-D-Glc or L-Rha-L-Rha disaccharides. Therefore, the O-specific side chain consists of D-glucose–L-rhamnose repeating units rather than a random distribution of these two monosaccharides.

Anomeric Configuration of the O-Specific Side Chain. Results of the above enzymatic reactions with D-1 and D-2 subfractions have already indicated that both D-glucose and L-rhamnose have the β configuration. This result was confirmed by the nmr spectrum of the O-specific side chain (Figure 8) which exhibited a proton peak at τ 5.0 also indicating the β-anomeric configuration (Whyte, 1971; Michell, 1970) for both D-glucose and L-rhamnose.

Structure of the O-Specific Side Chain. On the basis of these chemical, physical and enzymatic studies, we have concluded that the O-specific side chain of the nonchromogenic strain *S. marcescens* Bizio represents a macromolecular entity consisting of repeating units of a D-glucose–L-rhamnose disaccharide. The detailed structure and anomeric configuration of the O-specific side chain is shown in Figure 9. Since the molec-

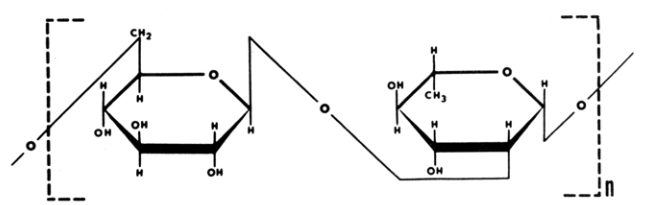

FIGURE 9: Structure of O-specific side chain from *S. marcescens* Bizio.

ular weight determined by the sedimentation–equilibrium method was 13,200, it was calculated that the analyzed O-specific side chain contains 43 repeating disaccharide units.

Discussion

Fractionation of the degraded polysaccharide from *S. marcescens* Bizio into the O-specific side chain and core confirmed the results of similar studies on the mild acetic acid hydrolysis of lipopolysaccharides from *E. coli* 071:K?:H12 (Müller-Seitz *et al.*, 1968), *Pseudomonas aeruginosa* 8602 (Fensom and Meadow, 1970), and *Shigella flexneri* 1b (I) (Romanowska and Lachowicz, 1970). It also demonstrated that the polysaccharide moiety of genus *Serratia* consists of same structural entities as those from *Salmonella* (Lüderitz, 1970), *Escherichia* (Heath *et al.*, 1966; Schmidt *et al.*, 1969), and *Shigella* (Simmons 1969). Furthermore, results of the present analytical and structural studies showed that the O-specific side chain of *S. marcescens* Bizio is a macromolecular compound consisting of repeating units of a simple D-glucose–L-rhamnose disaccharide. The average number of repeating units in the O-specific side chain was estimated to be 43. However, due to the inherent methodological limitations, it has not been possible to ascertain whether this value also represents the exact number of repeating units of O-specific side chain in intact endotoxin.

This report represents the first structural elucidation of an O-specific side chain from genus *Serratia*. Most of the repeating units studied to date consist of branched tetra- and pentasaccharides; the smallest unit thus far identified in some species of *Salmonella* (Robbins and Uchida, 1962) is a simple linear trisaccharide. Rapin and Mayer (1966) suggested that the O-specific side chain of the wild type of *E. coli* K-12 may consist of a L-rhamnose–D-galactose repeating unit; however, the structure of this disaccharide has not yet been established. Thus, the present study indicates that the O-specific side chains of Gram-negative bacteria may also be composed of simple disaccharide repeating units. So far, there are no reports indicating single sugars to be the only components of O-specific side chains.

The other more extensively studied naturally occurring disaccharides which contain a molecule of L-rhamnose and D-glucose are rutinose and neophesperidose, the carbohydrate moieties of the flavanone glycosides rutin, hesperidin, and neohesperidin. These two disaccharides differ only in the position of the linkage between L-rhamnose and D-glucose: rutinose is 6-*O*-L-rhamnopyranosyl-D-glucose, whereas neophesperidose is 2-*O*-L-rhamnopyranosyl-D-glucose (Horowitz and Gentili, 1963). One of the disaccharides (fraction D-2) isolated in the present study from the partial acid hydrolysate of O-specific side chain is thus similar to, if not identical with, rutinose. The uncertainty lies only in the assignment of the anomeric configuration. Although Zemplén and Gerecs (1934, 1935) originally suggested that in rutinose L-rhamnose has the β configuration, Horowitz and Gentili (1963) argued, solely on the basis of the optical rotation of methylated α- and β-L-rhamnosides, that in neophesperidose and by analogy in rutinose, L-rhamnose has the α configuration. Okada *et al.* (1963a) isolated from cultures of *Aspergillus niger* two enzymes called naringinase and hesperidinase based on their hydrolytic action on the flavonoids naringin and hesperidin. In subsequent studies on the substrate specificities of these two crystalline enzymes, Okada *et al.* (1963b) demonstrated that both enzymes were rhamnosidases; naringinase was found to be specific for β-1,4 and hesperidinase for β-1,6 linkages. The liberation of L-rhamnose from hesperidin and rutinose by the catalytic action of hesperidinase indicated a β rather than α configuration of L-rhamnose in this disaccharide. Since in our studies hesperidinase catalyzed the hydrolysis of fraction D-2, but not of fraction D-1, we concluded that in the former disaccharide L-rhamnose is linked in the β configuration to the C-6 glucose. A similar enzymatic treatment of fraction D-1 with α- and β-glucosidases established unequivocally that D-glucose also had the β configuration. By studying the nmr spectra of 14 common oligosaccharides, Whyte (1971) established that the anomeric protons on α-glycosidic linkages resonated at τ 4.35 $\pm$ 0.23 and the anomeric protons on β-glycosidic linkages resonated at τ 4.98 $\pm$ 0.05. Detection of a single anomeric signal at τ 5.0 confirmed the results of enzymatic studies and indicated clearly that in the O-specific side chain of *S. marcescens* Bizio both L-rhamnose and D-glucose have β configurations.

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Substrate Stereochemical Requirements in the Reductive Inactivation of Uridine Diphosphate Galactose 4-Epimerase by Sugar and 5′-Uridine Monophosphate†

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ABSTRACT: The reductive inactivation of uridine diphosphogalactose 4-epimerase (EC 5.1.3.2) by specific sugars in the presence of 5′UMP has been investigated as a model reaction for the first step in the epimerization of D-glucose to D-galactose. By following the stereospecific transfer of tritium label from sugar to enzyme, we have determined that the 4 position of D-galactose, but not D-glucose, is active in the reduction of enzyme. The 1 positions of both sugars are also active. The labeled compound dissociated from the enzyme was identified as B-[4-^{3}H]DPNH by its migration with DPNH on several chromatographic systems, as well as by incubation with specific dehydrogenases. A model is presented of the common stereochemistry of three isomeric centers around the position of hydrogen transfer in the sugar molecules that are active as model substrates.

Uridine diphosphate galactose 4-epimerase from *Escherichia coli* is a dimer containing a tightly bound DPN$^+$ (Wilson and Hogness, 1969) which catalyzes the intramolecular epimerization of UDP-D-galactose[1] to UDP-D-glucose (Glaser and Ward, 1970). Both yeast and *E. coli* epimerase have been shown to undergo a model reaction with free sugar and 5′UMP which results in the inactivation of enzyme and the net formation of tightly bound DPNH (Kalckar *et al.*, 1970; Bertland *et al.*, 1971). It has been speculated that this reaction represents the first step of the epimerization reaction (Davis and Glaser, 1971). It is reasonable to predict that in the model reaction the H from the 4 position of the sugar moiety is removed by DPN$^+$ in an oxidation–reduction reaction yielding DPNH and a 4-keto intermediate. Recent work utilizing 4-keto sugar nucleotide analogs (Nelsestuen and Kirkwood, 1971), as well as NaB^{3}H$_4$ trapping experiments in the presence of enzyme and UDP-galactose in which UDP-[4-^{3}H]hexoses were isolated (Maitra and Ankel, 1971), give strong support for the presence of a 4-keto intermediate in the normal reaction.

The relation of the model reaction to the complete one became ambiguous with the finding of Davis and Glaser (1971) that the reductive inactivation reaction shows an isotope effect upon incubation with [3-^{2}H]glucose, but not with [4-^{2}H]glucose. This implicated the 3 position in the epimerization. However, these workers observed no transfer of ^{3}H to enzyme upon incubation with [3-^{3}H]glucose.

Therefore, the object of these experiments was to test whether the proposed model reaction is reflective of the enzyme reaction, and if so, what are the stereochemical requirements of the enzyme for substrate. Also, we considered that, if the model reaction is indicative of the first step of the epimerization, then any other functional groups on the enzyme besides DPN$^+$ which might be involved in the reaction

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[1] Abbreviations used are: uridine 5′-(α-D-galactopyranosyl pyrophosphate), UDP-galactose; UDP-D-glucose 4-epimerase (EC 5.1.3.2), UDP-galactose 4-epimerase.