INFECTION AND IMMUNITY, July 1979, p. 85-92
0019-9567/79/07-0085/08$02.00/0

Vol. 25, No. 1

Structure and Serological Specificity of the K13-Antigenic Polysaccharide (K13 Antigen) of Urinary Tract-Infective *Escherichia coli*

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Received for publication 28 February 1979

The primary structure of the K13-antigenic polysaccharide (K13 antigen) of *Escherichia coli* O6:K13:H1 was elucidated by composition, periodate oxidation, Smith degradation, and methylation analysis. The polysaccharide consists of a repeating sequence of 3-linked ribofuranose and 7-linked 3-deoxymannooctulosonic acid (KDO). About 50% of the KDO residues are *O*-acetylated at position 4 or 5. Measurement of the optical rotary dispersion indicated that in aqueous solution the K13 polysaccharide assumes a secondary structure in which the carboxyl groups of KDO are engaged. The serological specificity of the K13 polysaccharide is expressed through KDO and its *O*-acetyl substituent, the ribose unit being antigenically silent. There are two populations of anti-K13 antibodies, one directed against the charged region of the KDO and the other against the *O*-acetyl groups.

An interest in *Escherichia coli* in recent years from both human and veterinary medicine has been followed by an interest in the surface structure of these bacteria because of their special role in pathophysiological processes, their usefulness in epidemiological studies, and their importance for the normal immunological status of the host. In view of the potential importance of the *E. coli* K antigens (14, 20) in infection and protection against infection (15), an investigation of their chemical structure was considered necessary.

E. coli antigens K1, K2, K12, and K13 are most frequently associated with urinary tract infections (14). These antigens are all acidic polysaccharides (22). The K1 polysaccharide is a homopolymer of *N*-acetylneuraminic acid linked α-(2-8) (19), and the K2 antigen is a polymer of galactose and glycerol phosphate (K. Jann et al., submitted for publication). The K12 antigen (unpublished data) and the K13 polysaccharide contain 3-deoxymannooctulosonic acid (KDO) as their acidic component. The occurrence of KDO as a major component of an extracellular polysaccharide of *E. coli* is unusual. Taylor reported the identification of KDO as the major component of an acidic polysaccharide isolated from a rough strain of *E. coli* (28), which was later serotyped as K6 (F. Ørskov, personal communication). Recently, the primary structure of the KDO containing capsular polysaccharide from *Neisseria meningitidis* 29e (2) was reported. In this communication we describe the isolation, structural elucidation, and serological properties of the *E. coli* K13 polysaccharide.

MATERIALS AND METHODS

Bacteria. *E. coli* O6:K13 strain F2677 was obtained from F. and I. Ørskov, Statens Seruminstitut, Copenhagen, and cultivated as previously described (6).

Polysaccharide preparations. The acidic polysaccharide of *E. coli* O6:K13 was isolated from liquid cultures as described by Gotschlich et al. (8). Briefly, the bacteria together with acidic polysaccharides are precipitated from the medium by the addition of cetyltrimethylammonium bromide (Cetavlon). The sedimented material is then extracted with calcium chloride. Purified polysaccharide is obtained by a sequence of steps involving alcohol precipitation and extraction with cold buffered phenol. Purified polysaccharide was also kindly supplied by J. B. Robbins, Bethesda, Md. Minor contaminants could be removed by chromatography on diethylaminoethyl-cellulose. The polysaccharide (450 mg) was dissolved in 0.05 M sodium acetate (pH 6.0) containing 0.2 M sodium chloride and applied to a column of diethylaminoethyl-cellulose (3 by 60 cm) equilibrated with the same buffer. The column was washed with 510 ml of starting buffer, and the polysaccharide was eluted with a gradient of 0.2 to 1.0 M sodium chloride in the same buffer.

De-*O*-acetylated polysaccharide was prepared by incubation of polysaccharide in 0.1 N sodium hydroxide for 4 h at 37°C. The incubation mixture was dialyzed against water and lyophilized. Periodate-oxidized and reduced polysaccharide was prepared as described previously (11). The K13 polysaccharide was esterified with ethyleneoxide (10) or methanolic HCl. The polysaccharide (20 mg) was stirred in 0.1 N methanolic HCl for 48 h at 4°C. The insoluble material was

washed with anhydrous methanol, dialyzed, and lyophilized (yield, 17 mg). The esterified polysaccharide was reduced with sodium borohydride in 10% glycerol (10).

Optical measurements. The optical rotation of the K13 polysaccharide (4 to 6 mg/ml) was measured with a Perkin-Elmer model 141 polarimeter at 23°C and 589 nm in either water, 0.1 HCl, or as indicated. No measurable effect was observed between 25 and 85°C at 365 or 589 nm. Optical rotary dispersion of the sodium salt of the polysaccharide was measured with a Cary spectropolarimeter in 0.5 cm cells at 27°C (2 to 3 mg of polysaccharide per ml).

Analytical methods. Thin-layer chromatography on cellulose, paper chromatography, and paper electrophoresis were performed as previously described (10). KDO was detected after chromatography and electrophoresis by the method of Anderson (1). Carbohydrate was detected in column effluents with the orcinol reagent (16). KDO was quantitated by the method of Waravdekar and Saslow (29), and ribose was determined as ribitol pentaacetate by gas chromatography (25) after hydrolysis of the polysaccharide in 2 N HCl for 3 h at 100°C. Oligosaccharides were derivatized in 0.1 ml of pyridine–trimethylsilylimidazole (4:1, vol/vol) (Trisil-Z, Pearce Co.) for 15 min at 23°C. The mixture was analyzed by gas chromatography on 5% OV-101 with a temperature program from 120 to 220°C at 4°C/min. Determinations of acetyl and phosphate were performed by I. Fromme (5). Ultracentrifugation was performed on 0.5% polysaccharide solutions in Sørensen buffer (pH 7.0) as described previously (10).

Partial hydrolysis of the polysaccharide. The polysaccharide (100 mg) was hydrolyzed in 10 ml of acetic acid for 1 h at 100°C. The hydrolysate was evaporated to dryness and fractionated on a column of Bio-Gel P-2 (2 by 150 cm) in 0.2 M ammonium acetate. The fractions were repeatedly lyophilized to remove ammonium acetate.

An oligosaccharide was prepared by de-*O*-acetylating 98 mg of polysaccharide in 10 ml of 0.1 N sodium hydroxide for 1 h at 37°C and acidifying the incubation mixture with Dowex 50×8 H^+-form. This solution was refluxed for 1 h at 100°C and lyophilized. The lyophilized material was fractionated on Bio-Gel P-2 as described above. Fractions eluting in the position of an oligosaccharide were pooled. The oligosaccharide was further purified by preparative electrophoresis in pyridine–acetic acid–water (10:4:86, vol/vol/vol) at pH 5.4 (10).

Oligosaccharides were prepared from periodate-oxidized polysaccharide by Smith degradation (7). The oxidized and reduced polysaccharide (25 mg) was hydrolyzed for 30 min in 0.01 N sulfuric acid at 100°C (4). The hydrolysate was neutralized with barium hydroxide and fractionated on a Bio-Gel P-2 column (2 by 150 cm) with ammonium acetate.

Methylation analysis. The polysaccharide was methylated by a modification (24) of the procedure described by Hakomori (9). The methylated product was first hydrolyzed in 90% formic acid for 3 h at 100°C and then in 0.25 N sulfuric acid for 16 h at 100°C and converted to alditol acetates. Oligosaccharide S4 obtained from periodate oxidation (see below) was methylated, and a portion was analyzed by gas-liquid chromatography and mass spectrum analysis without prior hydrolysis. The remainder was degraded with 0.4 N anhydrous methanolic HCl and analyzed as alditol acetates.

To establish the linkage of KDO, the polysaccharide was acetylated in acetic anhydride–pyridine (1:1) for 2 h at 55°C and methylated by the procedure described by Lindberg (17). The methylated product was purified on LH-20 (24) and subjected to mild acid hydrolysis in 1 N trifluoroacetic acid for 1 h at 100°C. The KDO released was reduced and then esterified with diazomethane in ether–methanol until a faint yellow color remained, and the solution was evaporated to dryness. The residue was acetylated and analyzed by gas chromatography on 5% OV-101 with a temperature program from 120°C at 8°C/min. For gas chromatography and mass spectrometry a 10% SE-30 column and the same temperature program were used.

Serological methods. Antiserum against formalized *E. coli* O6:K13 was used to characterize the polysaccharide as a K antigen by gel diffusion (26) and immunoelectrophoresis (22). The polysaccharide K antigen was shown to be acidic by agarose electrophoresis in one dimension and subsequent diffusion against Cetavlon as a precipitant, as described by Ørskov (21).

The polysaccharide was analyzed for its immunodeterminants by gel diffusion (26), quantitative precipitation, and precipitation inhibition (10, 26) of native, deacetylated, periodate-oxidized, and carboxyl-reduced polysaccharide.

RESULTS

Isolation of the polysaccharide. The acidic polysaccharide of *E. coli* K13 was isolated from liquid cultures by precipitation with Cetavlon and extraction of the sedimented material with calcium chloride as described by Gotschlich et al. (8). Purified polysaccharide was obtained from this extract by the method described for the purification of meningococcal capsular polysaccharides (6). Minor contaminants of protein, nucleic acid, and lipopolysaccharide could be removed by ion exchange chromatography of this material on diethylaminoethyl-cellulose as described above. The polysaccharide was eluted as a narrow peak and migrated as a single negatively charged component in agarose gel electrophoresis. This method of isolation differs from the procedure used to isolate *E. coli* antigens occurring in O-groups 8 and 9 (23). Attempts to purify K antigens of *E. coli* causing urinary tract infections from acetone-dried bacteria after growth on agar resulted in low yields.

Characterization of the polysaccharide. The sodium salt of the K13 polysaccharide exhibited a small negative specific rotation, $[\alpha]_D = -16$ (23°C in water), which gradually increased with decreasing pH. A small positive rotation not due to hydrolysis was observed in

0.1 N HCl. The optical rotary dispersion spectra of the K13 polysaccharide dissolved in water and 0.1 N HCl are compared in Fig. 1. There is a striking difference between the spectra of the K13 polysaccharide and free KDO. Analogous results were reported for *N*-acetylneuraminic acid and the K1 polysaccharide (13). The sodium salt of the K13 polysaccharide exhibits a maximum at 230 nm that is shifted to 245 nm in 0.1 N HCl. Similarly, the crossover was shifted to a longer wave length (209 to 214 nm) by acid. The absolute value of the minimum (207 nm) decreased and was shifted to a higher wave length (218 nm) in 0.1 N HCl.

The molecular size of the K13 polysaccharide was investigated by gel filtration and analytical ultracentrifugation. The polysaccharide was eluted from Sepharose 4B column with a relative elution volume of 0.5 and sedimented at 1.7S during ultracentrifugation. Some polysaccharide preparations contained a component with a higher apparent molecular weight when studied by either gel filtration or ultracentrifugation. Similar behavior was observed during analysis of *E. coli* K2 and K62 polysaccharides (Jann et al., submitted for publication). Since these components are chemically and serologically identical, they were not separated in the present study.

Ribose and KDO were detected in acid hydrolysates of the polysaccharide by thin-layer cellulose chromatography and paper electrophoresis. The acidic component was isolated from acid hydrolysates by ion exchange chromatography and shown to react with thiobarbituric acid to give an absorption spectrum identical to that expected for KDO. The polysaccharide consists of ribose, KDO, and *O*-acetyl in the molar ratio 1:0.9:0.4 (Table 1). After de-*O*-acetylation, the KDO values obtained were approximately double the values obtained before deacetylation. This indicates that about half the KDO residues are *O*-acetylated at position 4 or 5. Substitution at either position would prevent oxidation of KDO by periodic acid, thus reducing the color produced in the thiobarbituric reaction.

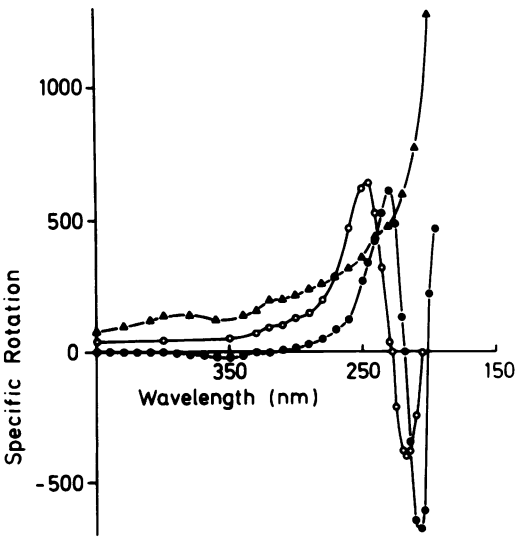


FIG. 1. *Optical rotary dispersion spectra of the K13 polysaccharide and KDO. The optical rotary dispersion spectrum of the sodium salt of the K13 polysaccharide was measured in water, c = 2.4 mg/ml (●) and in 0.1 N HCl, c = 2.1 mg/ml (○), and that of KDO was measured in water, c = 1 mg/ml (▲).*

Periodate oxidation. When the deacetylated polysaccharide was treated with periodate for 100 h at 4°C, practically all of the KDO and none of the ribose was destroyed. Subsequent reduction of the oxidized polysaccharide followed by total acid hydrolysis liberated erythritol. This indicates that ribose is bound in a linkage stable to periodate oxidation and that the KDO is glycosidically substituted at C7 or C8.

Smith degradation. To obtain oligosaccharides containing ribose, advantage was taken of the periodate sensitivity of the KDO residues in the polysaccharide. The K13 polysaccharide was oxidized with sodium periodate and reduced with sodium borohydride. After decomposition of the reagents, the mixture was dialyzed. The product obtained by lyophilization was partially hydrolyzed in 0.01 N sulfuric acid. The neutralized hydrolysate was chromatographed on Bio-Gel P-2. The elution pattern is presented in Fig. 2A. All oligosaccharide fractions (1 to 4) contained ribose and erythritol in the same relative amounts. Fraction 5 contained monomeric ribose. The oligosaccharides from fractions 3 and 4 were found to be homogeneous by gas chromatography of their trimethylsilyl ethers. Their retention times relative to lactose were 0.98 and 0.7, respectively. Both contain ribose and erythritol in a molar ratio of 1:1.6. Only oligosaccharide S4, obtained from peak 4, was analyzed further. These results indicate that during Smith degradation the K13 polysaccharide is

TABLE 1. *Composition of the K13 polysaccharide*

Component	Before alkali treatment		After alkali treatment[a]	
	%	Molar ratio	%	Molar ratio
Ribose	33	1	33	1
KDO	26	0.43	50.9	0.85
Acetyl	3.9	0.4	ND[b]	ND
Ash	9.8	ND	ND	ND

[a] After treatment with 0.1 N NaOH for 3 h at 37°C.
[b] ND, Not determined.

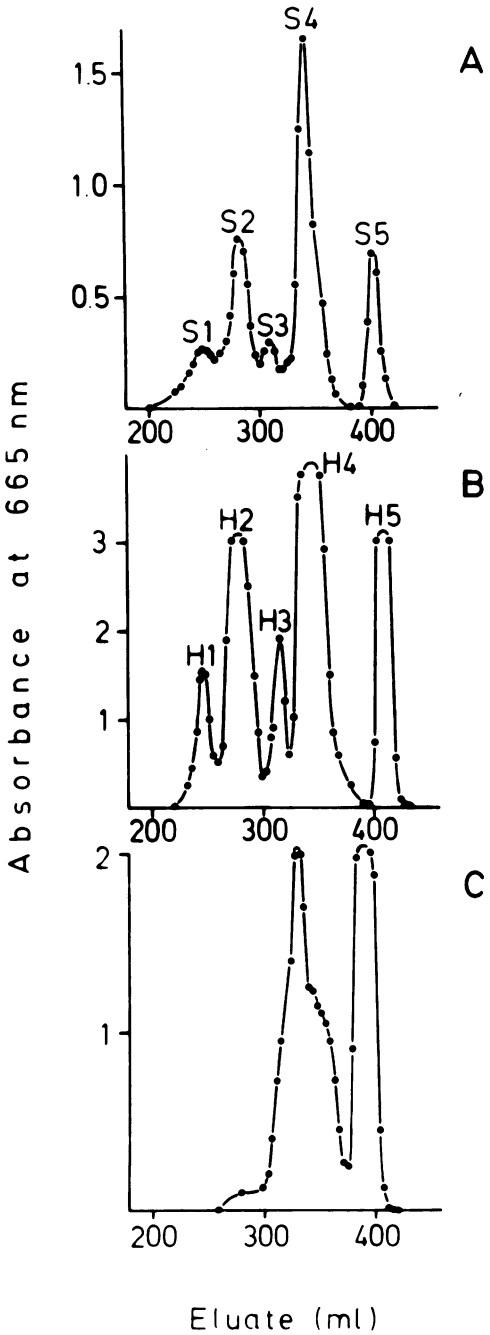


FIG. 2. *Fractionation on Bio-Gel P-2 of the K13 polysaccharide after various degradations. (A) Periodate-oxidized and borohydride-reduced polysaccharide; (B) polysaccharide hydrolyzed in 1% acetic acid; (C) deacetylated polysaccharide. Column size was 2 by 150 cm, elution was carried out with 0.1 N ammonium acetate, and fractions were analyzed with the orcinol reagent.*

split into fragments of various size, all containing ribose and erythritol. The higher-molecular-weight fragments found in fractions 1 and 2 seem to be due to incomplete hydrolysis and to be multiples of fragment S4.

Acid hydrolysis. The polysaccharide was hydrolyzed in 1% acetic acid, and the lyophilized hydrolysate was chromatographed on Bio-Gel P-2. The elution pattern was similar to that obtained after Smith degradation (Fig. 2B). Calibration of the column with oligosaccharides of the isomalto series indicated that the material from peak 1 was larger than an octasaccharide, that from peak 2 had the size of a penta- or hexasaccharide, and that from peak 4 had the size of a di- or trisaccharide. The material from all peaks contained ribose (30 to 32%) and KDO (35 to 37%). Paper electrophoretic analysis showed that peak 4 contained monomeric KDO and an oligosaccharide (H4) which had an electrophoretic mobility relative to KDO of 0.75. Like Smith degradation, the mild acid hydrolysis splits the polysaccharide into fragments of various sizes which seem to be multiples of the smallest unit (H4). Autohydrolysis of the acidic form of the deacetylated polysaccharide gave rise to only one oligosaccharide (Fig. 2C). On Bio-Gel P-2 this fragment eluted in the same region as fragment S4 (from Smith degradation) and oligosaccharide H4 from mild acid hydrolysis. No oligosaccharides containing only ribose were detected in the hydrolysates.

Methylation analysis. To analyze the substitution of the ribose moieties, the polysaccharide was subjected to methylation with dimethylsulfoxide–sodium borohydride–methyl iodide, as described previously (24). The product was hydrolyzed, reduced with sodium borohydride, and acetylated with acetic anhydride/pyridine. The resulting partially methylated ribitol acetates were analyzed by gas chromatography and mass spectrometry. The gas chromatogram is shown in Fig. 3, and the primary fragments obtained by mass spectrometry, together with the retention times and relative intensities of the corresponding peaks, are given in Table 2. By comparison with known data (3), the main peak can be ascribed to 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methylribitol. The minor peak exhibited by 1,4-di-*O*-acetyl-1,3,5-tri-*O*-methylribitol is probably derived from nonreducing ribose at the chain end. It is assumed that the triacetyl glycerol is a degradation product obtained during the methylation and subsequent hydrolysis, especially since it was not detected in hydrolysates of the unmethylated polysaccharide.

Methylated products of KDO are not detected in the above procedure for methylation analysis

probably because of destruction during methylation or subsequent work-up. Therefore we acetylated the polysaccharide prior to methylation to increase the yield of methylated polysaccharide and used mild acid hydrolysis to release the KDO residues. The keto function was reduced with sodium borohydride, and the carboxyl group was esterified with diazomethane. The KDO was thus partially recovered and analyzed as a partially methylated alditol actate of its methyl ester (Fig. 4). The primary fragments 45, 189, and 233 are consistent with peak B being 2,6,7-tri-*O*-acetyl-4,5,8-*O*-methyl-3-deocyoctonate methyl ester. Although the primary fragment [M minus CO_2CH_3] was not detected, a secondary fragment appeared which is consistent with the elimination of acetic acid from this primary fragment (27). The structure of this KDO derivative is further supported by the appearance of fragments 113, 129, 130, 173, and 174. These data indicate that the KDO residues are glycosidically substituted at C7.

Oligosaccharide S4, which contains erythritol as a degradation product of KDO, was also methylated. The erythritol residue contains that part of the KDO residue (C5-C8) that is substituted by ribose. The methylated product, which was homogeneous by gas chromatography, was subjected to mass spectrometric analysis without prior hydrolysis. The main fragments and the structure that can be derived from them are shown in Fig. 5.

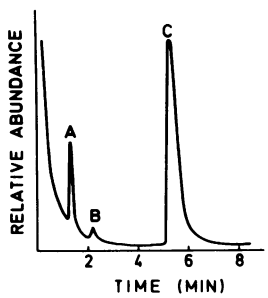


FIG. 3. *Gas chromatogram of the methylated K13 polysaccharide.*

The mass spectrum is consistent with that of per-*O*-methylated ribofuranosyl erythritol, as evidenced by fragments with *m/e* values of 147 and 175. The presence of ions with *m/e* 89 and *m/e* 45 and the absence of ions with *m/e* 133 indicate that the erythritol is substituted at C3. This corresponds to a substitution of KDO at C7 in the intact polysaccharide. When the methylated oligosaccharide S4 was subjected to methanolysis and *O*-acetylated, a component was obtained in subsequent gas chromatography which exhibited mass fragments characteristic of 3-*O*-acetyl-1,2,4-*O*-methylerythritol. These results indicated that the polysaccharide consists of a linear repeating sequence of 3-linked ribofuranose and 7-linked KDO. This is corroborated by methylation analysis of higher oligosaccharides obtained by mild acid hydrolysis (from peaks 1 to 3 of Fig. 2B). In these analyses, 3-substituted and terminal ribofuranose units were found with a decreasing amount of terminal ribofuranose as the oligosaccharides increased in size (data not shown).

Serological studies. The purified polysaccharide, as well as the *O*-antigenic lipopolysaccharide and a crude antigen extract of the same strain, was studied in immune electrophoresis using an antiserum against *E. coli* O6:K13 which contained anti-O6 and anti-K13 antibodies. As shown in Fig. 6, the polysaccharide showed one precipitation arc typical for K-antigenic acidic polysaccharides of low molecular weight, which is quite distinct from the precipitation arc given by *O*-antigenic lipopolysaccharides (22). The crude antigen extract showed both lines. This indicated that the acidic polysaccharide described here is the K13 antigen of *E. coli* O6:K13.

The importance of the constituents for the serological specificity of the K13 polysaccharide was tested by immune precipitation of the polysaccharide before and after chemical modifications. The results are presented in Figure 7. The intact polysaccharide exhibited a precipitation curve with two overlapping peaks, one of which was missing in the precipitation curve obtained with the deacetylated polysaccharide.

TABLE 2. *Retention times and mass spectrometric analysis of peaks obtained for alditol acetates of methylated polysaccharide*

Peak	RT[a]	Primary fragments					Intensity	Interpretation
		45	73	117(118)	145	161		
A	0.23		×		×		0.08	Tri-*O*-acetylglycerol
B	0.38	×		×		×	0.02	2,3,5-*O*-Methylribitol
C	0.89	×		×			1.0	2,5-*O*-Methylribitol

[a] Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

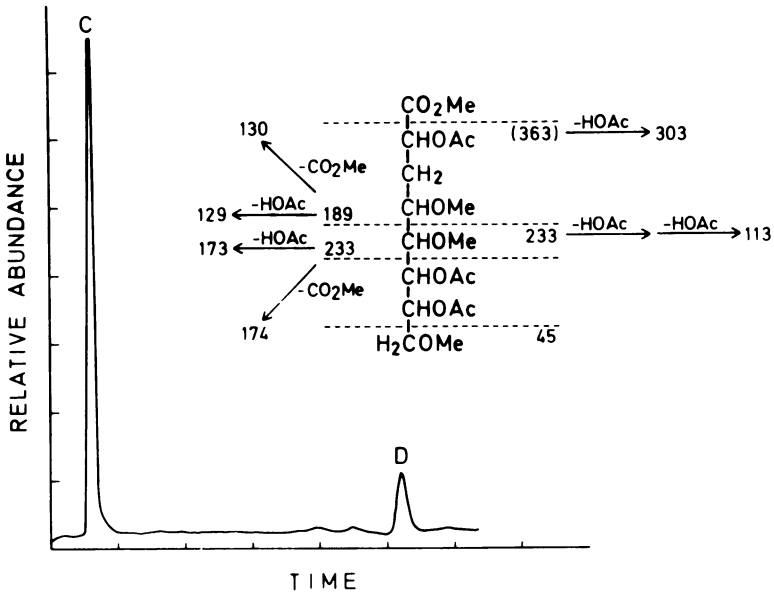


FIG. 4. *Gas chromatogram and fragmentation pattern of the methylated K13 polysaccharide after mild acid hydrolysis.*

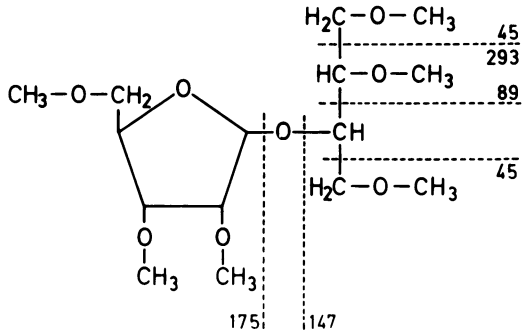


FIG. 5. *Fragmentation pattern of the methylated oligosaccharide S4 obtained by Smith degradation of the K13 polysaccharide. Only the characteristic primary fragments are shown.*

Periodate oxidation as well as carboxyl reduction abolished the serological activity of the polysaccharide. This indicated that there are two antigenic determinants in the K13 polysaccharide: the KDO residue with the carboxyl groups, which is dominant, and the *O*-acetyl substituent. In agreement with these results, preparations of periodate-oxidized or carboxyl-reduced oligosaccharide did not show precipitation arcs in immune electrophoresis.

DISCUSSION

The K13 antigen of *E. coli* O6:K13 belongs to a group of acidic polysaccharides with a relatively low molecular weight (22, 23). We found

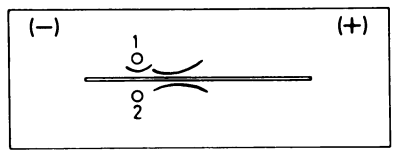


FIG. 6. *Immune electrophoresis pattern of crude (upper well) and purified (lower well) K13 polysaccharide obtained with O6:K13 antiserum (center trough).*

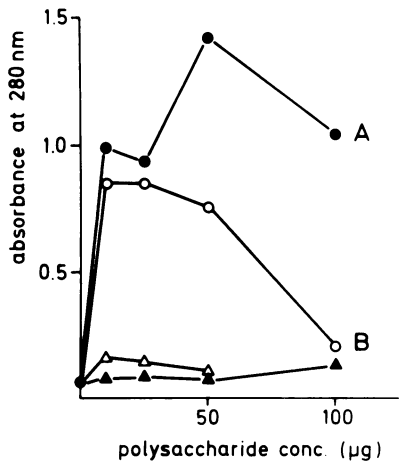
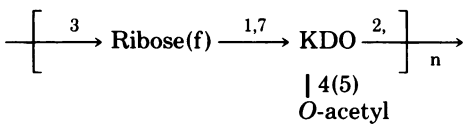


FIG. 7. *Quantitative precipitation curve of the acidic polysaccharide from E. coli O6:K13 antiserum. (●) K13 polysaccharide; (○) deacetylated polysaccharide; (△) periodate-oxidized polysaccharide; (▲) carboxyl-reduced polysaccharide.*

that it could not be obtained in reasonable yields from bacteria grown on agar, and we have therefore grown the bacteria in liquid culture and isolated the polysaccharide in pure form as described by Gotschlich et al. (8). The pure polysaccharide consists of equal amounts of ribose and KDO. About half of the KDO units are acetylated at positions 4 or 5. The presence of KDO complicated the structural analysis of the polysaccharide. To obtain a methylated derivative of KDO we have treated methylated polysaccharide with mild acid and converted the released KDO to its alditol ester. The mass spectrum observed is consistent with the fragmentation pattern expected for 2,6,7-tri-*O*-acetyl-4,5,8-*O*-methyl-3-deoxyoctonate methyl ester. The KDO moiety was also analyzed by conversion into erythritol residues by oxidation of the polysaccharide with sodium periodate, followed by reduction with sodium borohydride. The site of substitution by ribose remained intact during these reactions. Thus, Smith degradation was used for the fragmentation of the polymer. The intact polysaccharide and the main product of the Smith degradation (ribosylerythritol) were subjected to methylation analysis. Based on the information obtained on the products of gas chromatography and mass spectrometry, we propose the following formulation of the K13 polysaccharide:



Based on carbon-13 nuclear magnetic resonance experiments, a similar structure has been elucidated for the capsular polysaccharide of *N. meningitidis* (2). It consists of an alternating structure of 3-substituted *N*-acetyl-galactosamine and 7-substituted KDO acetylated at C4 or C5. The gas chromatogram of the methylated polysaccharide exhibited peaks due to 3-substituted and terminal ribofuranose in an approximate ratio of 50:1. Based on the disaccharide repeating unit, the molecular weight of the K13 polysaccharide chain can be roughly estimated as about 20,000. Ultracentrifugation and gel permeation chromatography indicated that in aqueous solution the polysaccharide is not homogeneous and seems to contain populations of various states of aggregation. This is in agreement with reports on capsular polysaccharide from *N. meningitidis* (18). Our measurements of the optical rotatory dispersion indicated a secondary structure of the K13 polysaccharide in which the carboxyl groups of KDO seem to play a role. Similar results were obtained with a polyneuraminic acid occurring in *E. coli* and *N. meningitidis* (12, 13). In the K13 polysaccharide, the carboxyl groups of the KDO residues must be accessible, since they represent the major antigenic determinants of this antigen. *O*-Acetyl groups also participate in the serological expression of the K13 polysaccharide. Since KDO is the site of acetylation, the KDO residue seems to be the center of serological specificity. Not all KDO residues are acetylated, and the situation is somewhat reminiscent of the expression of factors 4 and 5 in the *O*-antigenic lipopolysaccharide of *Salmonella typhimurium*. We can conclude from the present study that future attempts to increase the immunogenicity of the K13 polysaccharide by chemical modification should take into account the serological importance of the *O*-acetyl group and the carboxylate of KDO.

ACKNOWLEDGMENTS

We are grateful to Helga Kochanowski for the analytical ultracentrifugation and to Dietmar Borowiak for help with the mass spectrometry.

One of us (W.F.V.) is recipient of an Alexander von Humboldt Fellowship.

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