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Structure of the *Escherichia coli* K2 Capsular Antigen, a Teichoic Acid-Like Polymer

KLAUS JANN,* BARBARA JANN, M. ALEXANDER SCHMIDT, AND WILLIE F. VANN†

Max-Planck-Institut für Immunbiologie, Freiburg, West Germany

The primary structure of the K2 antigen of *Escherichia coli* was elucidated by composition, alkaline fragmentation, dephosphorylation with hydrofluoric acid, periodate oxidation, and methylation analysis. The polymer contains galactose in the pyranosidic and furanosidic ring form. It consists of phosphogalactopyranosyl glycerol and phosphogalactofuranosyl glycerol units in a molar ratio of 2:1. The sequence of these units is not known. The structure of the K2 antigen is reminiscent of that of certain teichoic acids of gram-positive bacteria. Using microprecipitation, it was shown that in the polymer galactoside is immunodominant.

In previous publications it has been shown that pathogenicity of *Escherichia coli* correlated with the chemical and physical properties of their polysaccharide antigens (24, 25). The polysaccharide K antigens most frequently associated with urinary tract infections, and notably with pyelonephritis, are K1, K2, K3, K5, K12, and K13 (9, 15). It was found that patients with urinary tract infections often develop antibodies against the causative *E. coli* strains (14, 16). In an animal model, anti-K antibodies were shown to be protective against experimental pyelonephritis (17). Since these capsular polysaccharides are not immunogenic per se in humans and animals (13, 17), they will have to be chemically modified to become immunogens. For such studies the knowledge of the polysaccharide structures is indispensable.

The K1 antigen is a homopolysaccharide consisting of α-2,8-linked *N*-acetylneuraminic acid (22), and the K13 antigen is a heteropolysaccharide with a repeating sequence of 3-linked ribose and 7-linked 2-keto-3-deoxymannosoctonic acid (30). In preliminary studies we have found that the K5 antigen consists of equimolar amounts of glucuronic acid and *N*-acetylglucosamine and that the K12 antigen consists of 2-keto-3-deoxymannosoctonic acid and L-rhamnose (unpublished data). In this communication we report the structure of the K2 antigen from *E. coli* O6:K2:H1.

MATERIALS AND METHODS

Bacteria and cultivation. *E. coli* O6:K2ac:H1 (strain Bi 7458-41 in the designation of the International Escherichia Centre, Statens Seruminstitute, Copenhagen) was used, which was originally isolated from the urine of a pyelonephritis patient. It was kept on D1.5 agar (29), and for the isolation of the polysaccharide it was grown at 37°C in DO medium containing Casamino Acids (2%), dialyzable portions of yeast extract (100 ml/liter, from 100 g) and glucose (0.2%) to the late logarithmic phase (4 to 6 h).

Isolation of the K2 antigen. The procedure described by Gotschlich et al. and Vann et al. (7, 31) was used, which is briefly described as follows. To precipitate the polysaccharide in the culture medium, cetyltrimethylammonium bromide was added to give a final concentration of 0.1%. The polysaccharide was extracted from the precipitate with 1 M calcium chloride. It was purified by three cycles of precipitation with ethanol (80%) and extraction with phenol buffered with sodium acetate to pH 6.5. The final aqueous phase was ultracentrifuged at 100,000 × g, dialyzed, and freeze dried. All operations were carried out at 4°C.

Analytical methods. Detection of sugar constituents by gas chromatography, paper chromatography, thin-layer chromatography, and paper electrophoresis, as well as the determination of neutral sugars, glycerol, and phosphate, have been described earlier (11, 12, 27). Analytical ultracentrifugation was performed in a Spinco model E ultracentrifuge with an An-H-Ti rotor at 50,000 rpm. Gas chromatographic analyses were run on a Varian Aerograph Series 1400, equipped with an autolinear temperature programmer and a Hewlett-Packard 3380 A integrator. A pH meter, model Radiometer Copenhagen pH M4, was used for titration, and a Perkin-Elmer polarimeter 141 was used for determination of optical rotations.

Periodate oxidation and Smith degradation. Periodate oxidation and Smith degradation (4, 6) were performed as previously described (26). The polymer was oxidized with sodium metaperiodate for 3 days at 4°C. Excess reagent was decomposed with ethylene glycol, and the product was reduced with sodium borohydride. The resulting mixture was dialyzed, and the product was obtained by lyophilization. For the determination of the constituents the oxidized polysaccharide was hydrolyzed at 100°C for 2 h in 2 N hydrochloric acid. The neutralized hydrolysate was prepared for gas chromatographic determination of

† Present address: Food and Drug Administration, Bethesda, MD 20014.

the oxidized products (11, 12, 27). To obtain oligosaccharides, the periodate-oxidized and subsequently reduced polysaccharide was hydrolyzed at 100°C for 1 h in 0.5 N sulfuric acid.

Fragmentation of phosphate linkages. The polymer was treated with 1 N sodium hydroxide for 16 h at 37°C under an atmosphere of nitrogen (1). After neutralization the fragments were dephosphorylated by treatment with alkaline phosphatase (12) and isolated by chromatography on Bio-Gel P2 and preparative paper chromatography in butanol-pyridine-water (6:4:3, by volume).

Dephosphorylating fragmentation with hydrofluoric acid. For direct dephosphorylation, the K2 antigen was kept in 55% aqueous hydrofluoric acid at 4°C for 3 days (27). The mixture was neutralized by dropwise addition of a saturated solution of lithium hydroxide at −15°C. The insoluble lithium fluoride formed was removed by centrifugation. The supernatant was deionized with Dowex 50 (H^+) and lyophilized. The fragments were fractionated on Bio-Gel P2, converted to their trimethylsilyl derivatives in pyridine-trimethylsilyl imidazole (4:1, by volume) for 15 min at 23°C (Trisil-Z, Pearce catalogue 1977), and characterized by gas chromatography on 5% OV-101.

Methylation analysis. Methylation analysis of the K2 antigen and its fragments was performed by a modification of the Hakomori method (8) as previously described (28). The mixture of partially methylated polyol acetates was analyzed by gas chromatography-mass spectrometry with a Finnigan model 3200 chromatograph-spectrometer.

Serological methods. The serological methods used in this study, i.e., gel precipitation, immune electrophoresis, and microprecipitation, have been described before (11). A modification of the immune electrophoresis (23), in which a 0.1% solution of cetyltrimethylammonium bromide is used instead of antiserum, was also employed.

Treatment with α- and β-galactosidases. One milligram of substrate in 0.5 ml of acetate buffer (pH 6.6) was incubated with a suspension of the enzymes in ammonium sulfate (10 μl of α-galactosidase from coffee beans [Boehringer] or 3 μl of β-galactosidase from *E. coli* [Boehringer]), and the mixtures were kept at 37°C. At the times indicated in Fig. 5, 50-μl samples were taken, heated for 1 min to 100°C, and, after the addition of 100 μl of ethanol, lyophilized. To the lyophilized samples, 25 μl of trimethylsilyl imidazole-pyridine (1:4, by volume) was added. After mixing, the samples were centrifuged, and the clear supernatants were analyzed by gas chromatography on OV-101 with a temperature program from 150 to 200°C (2°C per min).

RESULTS

Isolation and characterization of the K2 antigen. As described previously with the K13 antigen of *E. coli* (30), the bacteria were grown in a rich dialyzable medium (7, 31). In the isolation and purification of the K2 antigen, use was made of the insolubility of its cetyltrimethylammonium salt in water and of its calcium salt in 80% ethanol. After precipitation with cetyltrimethylammonium bromide, solubilization in dilute calcium chloride, three cycles of precipitation with ethanol, and extraction with cold phenol buffered with ammonium acetate (pH 6.8), traces of lipopolysaccharide were removed by ultracentrifugation. The supernatant was extensively dialyzed and lyophilized. The purified K2 antigen was obtained in a yield of 80 to 85 mg/liter of culture.

The K2 antigen had an optical rotation of $[\alpha]^{25}_D = +132.8$ ($c = 0.824$) in water and of $[\alpha]^{25}_D = +123.4$ ($c = 0.742$) in 0.1 N hydrochloric acid. Thus, in contrast to the K13 polysaccharide (30), the K2 polysaccharide has a pH-independent optical rotation. Although the polysaccharide exhibited one major peak in analytical ultracentrifugation ($s_{20,c} = 1.4 \times 10^{-13}$), the presence of a small amount of fast-sedimenting impurities interfered with the determination of the molecular weight. The titration curve of the polysaccharide showed only one neutralization step, which excluded the presence of phospho-monoesters.

The composition of the K2 antigen is shown in Table 1. It consists of equimolar amounts of galactose, glycerol, and phosphate. Whereas galactose was determined in the neutralized hydrolysates by gas chromatography after reduction with sodium borohydride and acetylation, glycerol was quantitated enzymatically (Boehringer, Mannheim) with the reduction step omitted. Attempts to determine both components simultaneously led to severe losses of glycerol during the manipulations.

Periodate oxidation and Smith degradation. Periodate oxidation of the polymer destroyed about 80% of the galactose and none of the glycerol. The analytical values obtained after periodate oxidation and reduction with sodium borohydride are also included in Table 1. Reduction of the oxidized K2 polysaccharide, followed by total acid hydrolysis, liberated threitol and the glycerol constituent of the polymer.

When the oxidized and reduced K2 antigen was hydrolyzed under the mild conditions used in Smith degradation, the results were the same as with the total acid hydrolysis described above. Only threitol and glycerol were obtained. In the neutralized hydrolysate that was not incubated with alkaline phosphatase before the gas chromatographic analysis (Fig. 1), only small amounts of threitol were found. Phosphatase treatment did not affect the amount of glycerol that could be detected.

The results of the periodate oxidation indicate that in the K2 antigen galactose is present either in the pyranosidic form linked at C4 or in the furanosidic form linked at C5.

Alkaline fragmentation. We wanted to

TABLE 1. *Composition of the K2 antigen before and after oxidation with sodium metaperiodate*

Prepn	Galactose		Glycerol		Phosphate	
	%	MR[a]	%	MR	%	MR
K2	56.6	1	23.5	0.8	9.9	1
K2 oxidized	11.0	0.2	24.5	0.8	10.5	1

[a] MR, Molar ratio.

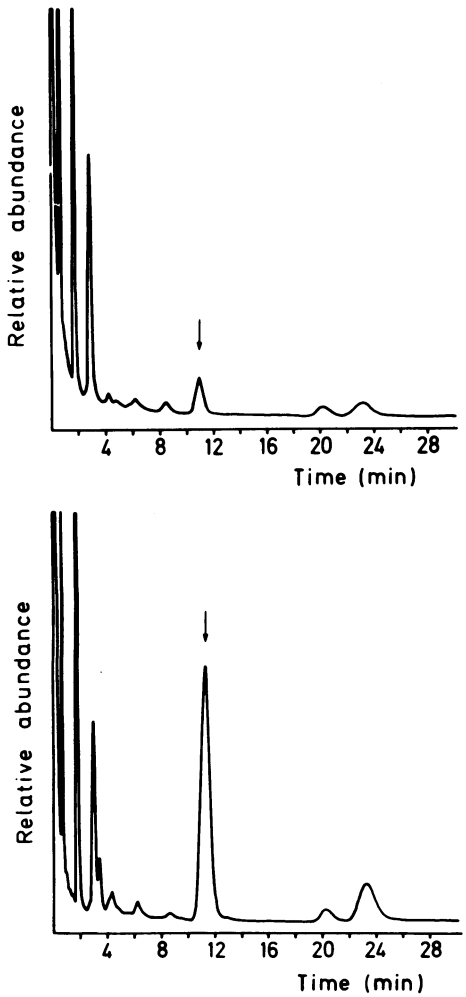


FIG. 1. *Gas chromatograms of Smith degradation products as alditolacetates before (top) and after (bottom) treatment with alkaline phosphatase (ECNSS-M, 150°C). The peak due to threitol is indicated by an arrow. The earlier peaks are due to impurities in the solvents.*

know whether glycerol phosphate is part of the main chain of the polymer, as in teichoic acids and related polymers (1), or whether it is a substituent of a main chain, as in the O100 antigen of *E. coli* (12). To differentiate between these possibilities and to gain information as to the position of the phosphate substitution, we subjected the polysaccharide to alkaline hydrolysis (1). Treatment with sodium hydroxide at 37°C overnight resulted in complete fragmentation. Electrophoresis of the neutralized and desalted mixture showed, after staining for phosphate, one spot with an electrophoretic mobility relative to galactose 1-phosphate of $M_{GP} = 0.84$. It could not be visualized with alkaline silver nitrate, which indicated that the material was not reducing. Only traces of glycerol phosphate and inorganic phosphate were detected in the degradation mixture. The main product was isolated by preparative paper electrophoresis. It could be dephosphorylated with alkaline phosphatase to a nonreducing component (fragment I) which stained with silver nitrate only after periodate oxidation. Galactose and glycerol were detected by gas chromatography in acid hydrolysates of this component. These results indicate that the K2 antigen is linear with a repeating sequence of phosphate-galactose-glycerol. Under alkaline conditions the phosphodiester bridge is cleaved in such a way that the phosphate group remains bound to galactose. This can only be the case if there is a free vicinal hydroxyl group available on the galactose but not on the glycerol. Thus the C2 position of glycerol must be substituted.

Dephosphorylating fragmentation with hydrofluoric acid. The phosphodiester bonds were selectively cleaved with concomitant removal of phosphate from the K2 antigen by treatment with 55% aqueous hydrofluoric acid at 4°C for 3 days. Longer incubations resulted also in partial cleavage of galactosyl bonds (data not shown). After removal of hydrofluoric acid from a 3-day incubation, only neutral glycosidically linked products were obtained. Paper chromatography showed only traces of galactose and a major product (fragment II) which could only be stained with periodate-silver nitrate.

Both fragments I and II were converted into their trimethylsilyl derivatives and subjected to gas chromatography. They both had a retention time, relative to lactose, of $RT_{lac} = 0.58$ when analyzed under identical conditions. Like fragment I, fragment II gave rise to galactose and glycerol on total acid hydrolysis. Thus the result of the dephosphorylating fragmentation with hydrofluoric acid (fragment II) is identical to that of the alkaline fragmentation followed by treatment with alkaline phosphatase (fragment I). In both cases the phosphate that bridges the galactosyl glycerol units in the polymer is removed.

Methylation and analysis. The results described above indicate that in the K2 antigen

galactose is bound to the 2-position of glycerol, which is linked via phosphate to the adjacent galactose unit. The sensitivity of galactose to periodate oxidation can be due to substitution at C4 in the pyranose form or at C5 in the furanose form. To establish the ring size of galactose and to confirm the substitution of glycerol at the 2-position, we performed a methylation analysis (28). Since methylations of polysaccharides are hampered by phosphate substituents, we analyzed fragment II, obtained by dephosphorylation with hydrofluoric acid.

The permethylated fragment II was purified by chromatography on Sephadex LH20. After hydrolysis, the product was reduced, acetylated, and subjected to gas chromatography. As shown in Fig. 2, the gas chromatogram exhibited two peaks (A and B) with relative intensities of 1:2. The retention time, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (tmg), is RT_{tmg} = 1.12 for peak A and RT_{tmg} = 1.21 for peak B. Table 2 shows the mass spectrometric fragments which are in accordance with the formulation of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylgalactitol for derivative A and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol for derivative B. Thus derivative A arose from nonreducing terminal galactofuranose, and derivative B arose from nonreducing terminal galactopyranose. Their relative ratios of 1:2 indicate that fragment II, and by the same token also fragment I, is a 1:2 mixture of galactofuranosyl glycerol and galactopyranosyl glycerol. As the methylated glycerol is very volatile, it was probably lost during the work-up of the methylation procedure.

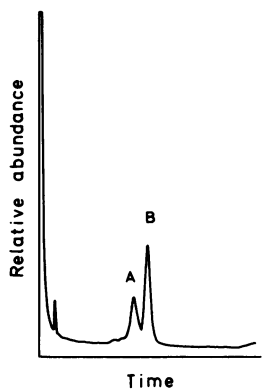
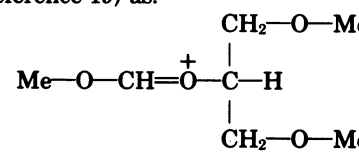


FIG. 2. *Gas chromatogram of partially methylated alditol acetates from fragment II (see text) (ECNSS-M, 150°C).*

When the methylated fragment II was directly subjected to gas chromatography without prior hydrolysis, one peak was obtained. This was not symmetric, probably due to insufficient separation of the galactopyranosyl and galactofuranosyl glycerol derivatives. The mass spectrum taken at peak maximum (Fig. 3) exhibited a strong signal at *m/e* 163. It is indicative of fragmentation of the galactose between C1 and C2, followed by rearrangement and secondary fragmentation to a product which contains only C1 of galactose and all three carbon atoms of the glycerol residue and which can be formulated (see reference 19) as:



Signals at *m/e* 89 due to the fragment CH(O-Me)-CH_2-O-Me and at *m/e* 59 due to the loss of formaldehyde from the former fragment (20) were absent from the mass spectrum. All other signals are those usually found with ring structures of permethylated sugars (19). The fragment *m/e* 45 was the base peak. For the other signals the nomenclature suggested in reference 19 can be applied: *m/e* 219 (A^1), *m/e* 187 (A_2^2), *m/e* 163 (J_1), *m/e* 111 (E_4), *m/e* 103 (A_1), *m/e* 101 (F_1, G_1), *m/e* 75 (J_1^1), and *m/e* 73 (H_2). The intense signal at *m/e* 71 is probably due to loss of *m/e* 30 (CH_2O) from fragment *m/e* 101. The very high ratio of *m/e* 187 to *m/e* 176 suggests an α-glycosidic bond (19), as was corroborated by later evidence.

The data indicated that there were no vicinal methoxy groups on the glycerol of the methylated fragment II. These results, in corroboration with those of the alkaline degradation of the K2 antigen, showed that in the polymer galactose is linked to the 2-position of glycerol.

TABLE 2. *Retention times and mass spectrometric analysis of peaks A and B obtained from methylated fragment II (Fig. 2)*

Peak	RT_{tmg}[a]	Ratio	Primary fragment (*m/e*)							Interpretation
			43	45	89	117	161	205	277	
A	1.12	1	+	+	+	+		+	+	1,4-Di-*O*-acetyl-2,3,5,6-tetra-*O*-methylgalactitol
B	1.21	2	+	+	+	+	+	+		1,5-Di-O-acetyl-2,3,4,6-tetra-*O*-methylgalactitol

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

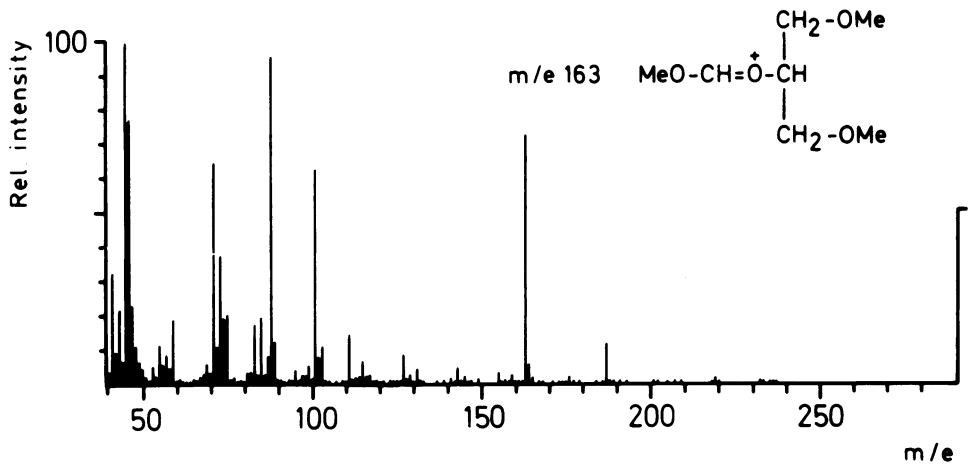


FIG. 3. *Mass spectrum of 2,3,4,6-tetra-O-methyl-D-galactopyranosyl-α-(1,2)-1,3-di-O-methyl-glycerol. Signal identifications are given in the text.*

Anomeric configuration of the galactosyl linkage. To establish the configuration of the galactosyl linkage, galactosyl glycerol (fragment II) was incubated with α-galactosidase and with β-galactosidase in ammonium acetate buffer at pH 6.6. After various times, samples were taken and heated briefly to 100°C to stop the enzyme action. After trimethylsilylation, the compounds were analyzed by gas chromatography. From the gas chromatograms the areas of substrate (galactosyl glycerol) and product (galactose) were integrated, and the ratio of substrate area to product area was plotted at various times. As shown in Fig. 4, galactosyl glycerol was rapidly and practically completely hydrolyzed by α-galactosidase. In contrast, β-galactosidase showed no effect on the same substrate. From these results, fragment II can be formulated as α-galactosyl glycerol. Since fragment II is a 2:1 mixture of glyceryl galactopyranoside and -furanoside, our interpretation implies that the α-galactosidase from coffee beans also splits the α-glyceryl galactofuranoside. Since we have found no literature citation referring to this problem, and since α-galactofuranosides were not available to us for model experiments, the formulation of an α-galactofuranoside in the K2 polymer may be considered as tentative. Fragment II had an optical rotation of $[\alpha]^{25}_{D} = +138°$. This corroborates the results of the enzymatic studies.

Serological studies. In immune electrophoresis with anti-OK and anti-K antisera, the precipitation arcs indicated that the relatively fast anodically moving polymer was the K2 antigen of *E. coli* O6:K2:H1. The presence of the K2 antigen could also be demonstrated when cetyltrimethylammonium bromide was used instead

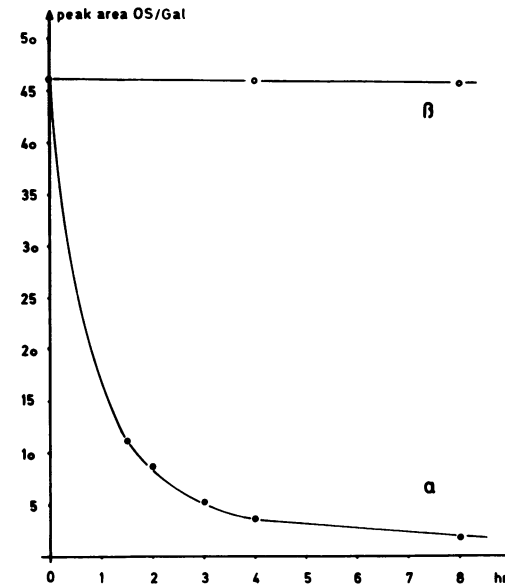


FIG. 4. *Cleavage of the galactosyl-glycerol bond of fragment II by incubation with α- (●) and β-galactosidase (○) expressed as peak area ratio of the respective trimethylsilyl derivatives.*

of antiserum in the second dimension. This simple and economic technique (23) was used for the identification of the K2 antigen preparations during purification.

Quantitative microprecipitation of the K2 antigen before and after periodate oxidation (Fig. 5) showed that its serological specificity was completely abolished by oxidation. The α- and β-methylgalactopyranosides, as well as fragment II (α-galactosyl-2-glycerol), did not inhibit the

precipitation reaction in amounts up to 2 μmol. These results indicate that intrachain galactose, substituted at C4 or possibly galactose 4-phosphate, is the major part of the K2 determinant.

DISCUSSION

The K2 antigen belongs to a group of *E. coli* K antigens which occur in bacteria causing urinary tract infections such as pyelonephritis (9), and which have relatively low molecular weight and relatively high charge density (24, 25). Like the other K antigens from urinary tract infective strains (K1, K3, K5, K12, and K13), the K2 antigen is only obtained in very poor yields when the respective bacteria are grown on agar. We have therefore grown *E. coli* O6:K2:H1 in a rich dialyzable medium and isolated the K2 antigen with the use of cetyltrimethylammonium bromide as described previously (11, 30, 31).

As established by alkaline degradation and dephosphorylating fragmentation with hydrofluoric acid, the K2 antigen consists of a repeating sequence of galactose, glycerol, and phosphate.

By dephosphorylating fragmentation, a main product was obtained which, by methylation and mass spectrometry as well as by the action of α- and β-galactosidases, could be identified as a 2:1 mixture of α-galactopyranosyl 1,2-glycerol and α-galactofuranosyl 1,2-glycerol. These are the structural units of the K2 antigen, and are joined by phosphodiester bridges between a primary alcohol group of the glycerol residues and the galactose residues. As evidenced by periodate oxidation and product analysis, the galactopyranosyl residues are substituted at C4 and the galactofuranosyl residues are substituted at C5. These data allow the formulation of the K2 polymer only in general terms without defining a sequence in the polymer, such as indicated in Fig. 6, in which Galp symbolizes galactopyranose, Galf represents galactofuranose, Glyc represents glycerol, and P represents phosphate. The pyranosidic and furanosidic ring forms of the galactose residues may either alternate regularly as →Galp→Galp→Galf→, or in any other sequence. The sites of fragmentation by alkali and hydrofluoric acid are also shown in Fig. 6.

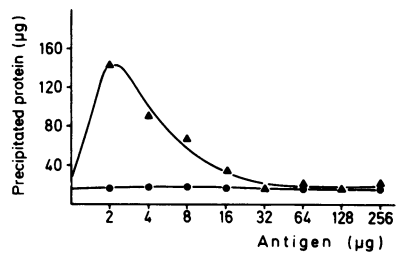


FIG. 5. *Microprecipitation of K2 antigen before (▲) and after (●) oxidation with sodium metaperiodate with rabbit OK-serum.*

The structure of the K2 antigen resembles that of teichoic acids, which are cell wall antigens of gram-positive bacteria (1, 18). The teichoic acids were originally thought to be polyglycerol or polyribitol phosphates which sometimes have carbohydrate substituents on the polyol residues. It was later found that this group of polymers is very complex and that sugar or amino sugar residues or even oligosaccharides may be part of the polymer chain. In the glycerol-containing hetero-teichoic acids, glycerol is usually substituted by sugar units at one of the primary alcohol groups, the other being substituted by phosphate. As shown in Fig. 6, the substitution of the glycerol units is different in the K2 antigen. A polygalactosylglycerol phosphate, together with a polyglucosylglycerol phosphate, was reported to occur in *Bacillus licheniformis* ATCC 9945 (3). To our knowledge this is the only polymer with the same sequence as the K2 antigen, albeit with a galactosyl 1,3 (or 1,1)-glycerol linkage. A somewhat similar polymer was isolated from *Bacillus coagulans* (5) for which a statistical repeating unit was presented consisting of galactosyl 1,2-glycerol 1-phosphate with a glucosyl substituent on the intrachain galactose.

It is interesting that such teichoic acid-like substances occur not only as cell wall antigens of gram-positive bacteria but also as capsular

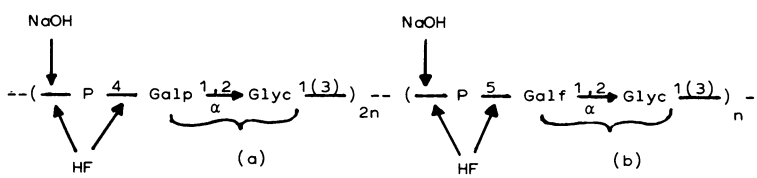


FIG. 6. *Structure of the K2 antigen of E. coli. The points of cleavage by hydrofluoric acid (HF) and sodium hydroxide (NaOH) are indicated by arrows. The α-galactofuranoside linkage is given in parentheses because it was inferred from the data of Fig. 4 and could not be tested on model compounds for lack of their availability. Fragment I, obtained by alkaline degradation followed by enzymatic dephosphorylation, and fragment II, obtained by dephosphorylation with HF, are identical. They are 2:1 mixtures of (a) and (b).*

antigens of a gram-negative bacterium. Although some pneumococcal capsular antigens also contain glycerol phosphate or ribitol phosphate in the polymer chain, these constituents are separated by larger oligosaccharide units (10). The K2 antigen is serologically closely related to the K62 antigen of *E. coli*. We are currently also analyzing this polymer (Jann et al., in preparation). It would be interesting to see whether the K2 antigen of *E. coli* is synthesized by the cell in a way analogous to that of the cell wall teichoic acids of gram-positive bacteria, which have recently gained new interest with respect to biosynthesis and regulation (2, 21).

Microprecipitation with anti-K2 antibody before and after periodate oxidation of the K2 antigen showed that, in the polymer, galactose is immunodominant.

No differentiation was made between the participation of furanosidic and pyranosidic forms of galactose in the serological expression of the K2 antigen. These facts are in accordance with the usual dominance of intrachain carbohydrate constituents over the polyol part in teichoic acids and related antigens, especially when the antisera were raised with whole bacteria carrying the polymers (18). In contrast, glycerol phosphate, which is a substituent on a polysaccharide chain, has been found to be immunodominant, e.g., in the O100 antigen (12).

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