JOURNAL OF VIROLOGY, Sept. 1975, p. 581-590

Vol. 16, No. 3
Printed in U.S.A.

Escherichia coli Capsule Bacteriophages

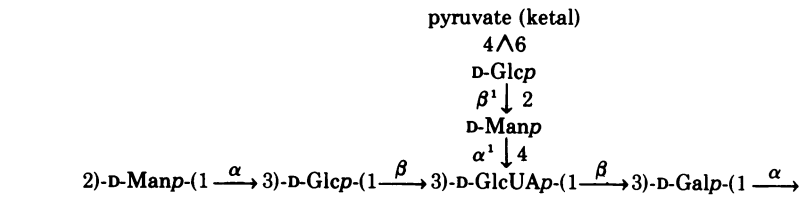
VI. Primary Structure of the Bacteriophage 29 Receptor, the *E. coli* Serotype 29 Capsular Polysaccharide

YUEN-MIN CHOY,[1] FRANK FEHMEL, NORBERT FRANK, AND STEPHAN STIRM*

Max Planck-Institut für Immunbiologie and Institut für Makromolekulare Chemie der Universität, Freiburg, Germany

Received for publication 19 February 1975

Using periodate oxidation, methylation analysis, characterization of oligosaccharides obtained by Smith degradation or partial acid hydrolysis, as well as proton magnetic resonance, the primary structure of the *Escherichia coli* serotype 29 capsular polysaccharide (the receptor of *E. coli* K phage 29) was reinvestigated. The polymer was found to consist of hexasaccharide repeating units of the following structure:



Like the other *Escherichia coli* capsule bacteriophages (38, 41), phage 29 is specific for the host capsular polysaccharide, i.e., it does not adsorb to acapsular host mutants and generally not to related strains with capsules of different serotype and chemical structure.

There is also a host capsule depolymerase activity associated with the particles of phage 29 (39)—small virions consisting of an isometric head with a base plate and a set of spikes (32, 41)—the active center of which is part of the virus spikes (4, 32). Within these organelles, the enzyme is part of subunits that contain polypeptides of about 57,000 daltons only (32). In the capsular glycan of the phage 29 host [*E. coli* Bi161/42 (24), 09:K29(A):H$^-$, also the serological test strain for the *E. coli* K29 antigen (25)] the virus particles catalyze the liberation of reducing glucose (39, 40).

The primary structure of the *E. coli* K29 capsular polysaccharide has been investigated by Nhan et al. (30; L. B. Nhan, Ph.D. thesis, University of Freiburg, 1970). However, the results of subsequent analyses of the products obtained from it by phage 29 action (F. Fehmel, Ph.D. thesis, University of Freiburg, 1972; see also [13]) were in partial disagreement with the structure proposed by these authors. Therefore, and because most of the anomeric configurations have not been elucidated by Nhan et al., we have reinvestigated the *E. coli* serotype 29 capsular polysaccharide.

[1] Present address: Department of Biochemistry, Chinese University of Hong Kong, United College, Hong Kong.

MATERIALS AND METHODS

The media and general techniques were those described and cited previously (41).

Bacterial strain. *E. coli* Bi161/42 [09:K29(A):H$^-$] (24), the host of *E. coli* capsule bacteriophage 29 (41) and also the serological test strain for the *E. coli* K29 antigen (25), was used. It was checked for K agglutination (25) with an *E. coli* OK 29 serum, kindly supplied, together with the strain, by I. Ørskov and F. Ørskov, World Health Organization International Escherichia Center, Statens Seruminstitut, Copenhagen.

Isolation and homogeneity controls of K29 capsular polysaccharide. *E. coli* Bi161/42 was grown on $D_{1.6}$ agar plates and extracted with phenol-water (43), and the capsular polysaccharide (as the sodium salt) was isolated from the water phase by fractional Cetavlon precipitation (0.3→0.06 M NaCl) after sedimentation of the cell wall lipopolysaccharide (21, 22, 30). To remove residual RNA, the product was digested with pancreatic RNase (21). Two hundred milligrams of dry bacteria, and therefrom 15 mg of the final lyophilized product, were obtained from one 14-cm agar plate.

The sedimentation of the polysaccharide (before and after mild alkali treatment, see below) in an analytical ultracentrifuge was carried out with the equipment previously described (32), and the agar gel double diffusion was performed according to Ouchterlony (22, 31). A 10-μl portion of a 1:2 dilution series of a 0.2% (wt/vol) polysaccharide solution in phosphate-buffered, physiological saline of pH 7.3 to 7.4, as well as 10 μl of an undiluted rabbit serum against formalin-killed *E. coli* Bi161/42 (OK serum, obtained by the standard procedure [25], K agglutination titer = 1:320) were applied to holes in 1% agarose gels on microscope slides. The slides were stored for 3 to 4 days in a moist chamber, and the precipitation arcs were washed and stained with amido black (compare description of immunoelectrophoresis [32]).

Derivatives of K29 polysaccharide. (i) For mild alkali treatment, a solution of the polymer in 0.25 N aqueous NaOH was warmed to 56 C for 6 h (compare [30]). (ii) For selective removal of the pyruvate ketal substituents, a 0.2% (wt/vol) solution of K29 polysaccharide in 0.125% aqueous oxalic acid was heated to 100 C for 2 h. Upon neutralization with dilute aqueous NaOH in the cold, the solution was dialyzed and lyophilized (yield: 82% [wt/wt]). (iii) For the reduction of carboxyl groups, the acidic form of native or depyruvylated polysaccharide was esterified with ethylene oxide and then reduced with sodium borohydride, following the experimental details given by Hungerer et al. (20). As determined by the carbazole-sulfuric acid method (12), 80 to 85% of the D-glucuronic acid (GlcUA) residues were reduced in this manner (yield: 88% [wt/wt]).

Qualitative and quantitative constituent analyses. For qualitative analyses of the constituents in K29 polysaccharide, its derivatives, or split products, the materials in 2 N HCl were hydrolyzed for 18 h at 100 C, and the acid was then removed by evaporation. Descending paper chromatography (PC) on Whatman no. 1 paper with ethyl acetate-pyridine-water (4/1/1, vol/vol/vol, solvent A) as an irrigant was used for the identification of neutral sugars, and high voltage paper electrophoresis (PE)(26) (45 V/cm, 1 to 3 h) in pyridine-glacial acetic acid-water (5/2/43, pH 5.2) on Schleicher & Schüll paper no. 2043a was used for the recognition of the acidic components GlcUA and pyruvic acid (mobility relative to GlcUA = 2.05). The staining method of Trevelyan et al. (42) was employed. Pyruvic acid was also identified as its dinitrophenyl hydrazone as follows. A 1% (wt/vol) solution of the polysaccharide in 1 N HCl containing 4% dinitrophenyl hydrazine was heated to 100 C for 3 h. After neutralization with 1 N NaOH, the mixture was extracted with ethyl acetate, the extract was evaporated, and the residue was subjected to PE together with suitable standards (conditions as above, no staining).

For the quantitative determination of neutral sugars, the method of Sawardeker et al. (34) was used: 1 ml of hydrolyzate, containing 1 to 15 μmol of hexoses, was neutralized with dry Amberlite IR 410-HCO_3^-, and xylose was added as an internal standard. $NaBH_4$ (2 to 20 mg) in 0.01 N NaOH was admixed, and the mixture was neutralized with 5% aqueous acetic acid after storage at 4 C overnight. The sodium ions were removed with dry Amberlite IR 120/H^+, and the boric acid was chased by repeated evaporation with methanol. The dry alditols were taken up in 0.5 ml of pyridine-acetic acid anhydride (1/1, vol/vol), and the mixture was heated to 100 C for 15 min. Upon evaporation to dryness, the alditol acetates were dissolved in chloroform and analyzed by gas-liquid chromatography (GLC), employing a Varian aerograph (model 1502, equipped with a flame ionization detector, and digital integrator, model 477), glass columns (80 by 1/8 inch [203 by 0.32 cm]) filled with 3% (wt/wt) ECNSS-M on Chromosorb G (80 to 100 mesh), a starting temperature of 165 C and a temperature increment of 0.5 C/min, and nitrogen (30 ml/min) as a carrier gas. After evaporation of HCl hydrolyzates, the pyruvate was determined with lactate dehydrogenase from hog muscle (Boehringer, no. 15373) as described by Czok and Lamprecht (10). Glucuronic acid and acetyl were directly estimated in the polymers with the carbazole-sulfuric acid method (12) and the procedure of Ludowieg and Dorfman (29), respectively.

Periodate oxidation and Smith degradation. The periodate consumption of K29 polysaccharide (dried in vacuo over phosphorus pentoxide at 37 C for 18 h) was determined by the method of Avigad (1).

For Smith degradation (14), an equal volume of 0.02 M aqueous sodium metaperiodate was added to a 0.2% (wt/vol) solution of the polymer, and the mixture was stored at 4 C in the dark for 4 h or for 7 days. After addition of 0.02 volumes of ethylene glycol and 30 min at room temperature, the solution was dialyzed against distilled water. Excess sodium borohydride was then added, and the mixture was kept at room temperature for 8 h and dialyzed again. Residual borohydride was removed by Amberlite IR 120/H^+ and evaporation with methanol. For the identification of the alditols produced, a part of the material was hydrolyzed with 2 M aqueous trifluoroacetic acid (TFA) for 1 h at 95 C. Upon evaporation, the mixture was acetylated and analyzed by GLC as described above, keeping the column temperature, however, at 110 C (for glycerol triacetate) or at 130 C (for erythritol tetraacetate). For the isolation of the major oligosaccharide (I1) formed, the material obtained after 7 days of oxidation and subsequent reduction was hydrolyzed for 16 h at room temperature with 0.25 N H_2SO_4, neutralized with barium carbonate, and concentrated and subjected to preparative PE using the same conditions as described above. The main oligosaccharide (mobility relative to GlcUA = 0.38) was eluted from the paper, checked for homogeneity by PC (with *n*-butanol-glacial acetic acid-water, 2/1/1, vol/vol/vol, solvent *B*; R_f value relative to lactose = 0.94), and evaporated to dryness (yield: about 19% [wt/wt] from K29 polysaccharide [$[\alpha]_D^{25}$ = +44°, c = 2.0, water]).

Methylation analyses. K29 polysaccharide, its derivatives, and split products were permethylated by the method of Hakomori (16), following, with minor modifications, the experimental details given by Hellerqvist et al. (18). Samples (10 to 80 mg) in 10-ml serum bottles were dried for 16 h in vacuo at 37 C over

phosphorus pentoxide and sealed with the rubber caps. Solutions of methylsulfinyl carbanion were prepared by adding 4.6 ml of dry dimethyl sulfoxide to 460 mg of sodium hydride in the same bottles, sealing, agitating for 30 min at room temperature with a magnetic stirrer (or, alternatively, in an ultrasonic bath), and, finally, warming to between 65 and 70 C for an additional hour. By means of a syringe, 3-ml portions of the methylsulfinyl carbanion solution were then transferred to each sample bottle, and the mixtures were agitated for around 8 h (polymers) or 1 h (reducing oligosaccharides). Up to this stage, all operations were carried out under protection by dry nitrogen. The solutions were then frozen in an ice bath, and 1.5 ml of methyl iodide was added slowly with shaking. After 1 to 8 h of agitation (until neutral), excess methyl iodide was evaporated, and the mixtures were poured into water and extracted with chloroform. Most methylated polymers were purified by passage over a Sephadex LH 20 column (length, 30 cm; area, 5 cm^2; elution with ethanol-chloroform, 2/1, vol/vol, at 20 ml/h), and the products (in chloroform) were checked for permethylation by infrared spectroscopy (Perkin Elmer, model 137 instrument; no absorption between 3,400 and 3,600/cm) and evaporated.

For hydrolysis, the methylated products were warmed to 70 C in 90% formic acid for 45 min, evaporated, and then heated to 100 C in 0.25 N H_2SO_4 for 14 h. Upon neutralization, the solutions were evaporated.

The identification of the *O*-methyl-aldoses obtained was performed by (i) PC of the free aldoses (37), (ii) GLC of the aldose acetates (3, 9), and (iii) GLC-mass spectrometry (MS) of the alditol acetates (5–7, 28), using suitable standards (available were: 2.3-Glc [2,3-di-*O*-methyl-D-glucose], 2.4.6-Glc, 3.4.6-Glc, 2.3.4.6-Glc, 2.3-Man, 2.4.6-Man, and 3.4.6-Man). (i) Butanone, saturated with 1% aqueous ammonia (solvent C), served as an irrigant; the chromatograms were sprayed with aqueous *p*-anisidine (1.5%)-trichloroacetic acid (5%) and heated to 100 C. (ii) Larger amounts of the methyl-aldoses with an R_f of 0.26, or of between 0.50 and 0.58, as obtained from native K29 polysaccharide, were purified by preparative PC, acetylated, and subjected to GLC (at 150 C) as described above. (iii) The mixtures of methylated aldoses, obtained by permethylation and hydrolysis, were reduced with sodium borohydride and acetylated as described above. The retention times of the alditol acetates produced were determined at 170 C, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T = 1.00), and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol (T = 5.39) as internal standards. The mass spectrometry was carried out with a combined GLC-MS instrument (Perkin Elmer, model 270B, equipped with a Honeywell visicorder, model 3508). Again, ECNSS-M columns (starting temperature: 165 C, temperature increment: 1 C/min) were employed, but with helium as a carrier gas. The ionization potential was 70 eV, the ionization current was 80 μA, and the temperature of the ion source was 180 C.

Isolation of oligosaccharides after partial acid hydrolysis. For the isolation of aldobiuronic acid (S2), 82 mg of K29 polysaccharide was hydrolyzed with 2 M TFA for 4 h at 95 C. Upon evaporation, the residue was taken up in water, and the acidic split products were adsorbed to an Amberlite IR 410/HCO_3^- column (length, 6 cm; area, 1.5 cm^2). After washing with water, they were eluted with 10% aqueous formic acid and evaporated to dryness. The fractions containing the aldobiuronic acid were checked for homogeneity by PC (solvent B, R_f value relative to lactose = 1.0) and PE (mobility relative to GlcUA = 0.68) (yield: about 17% [wt/wt] from the polymer).

For the isolation of the neutral oligosaccharide (SN), 200 mg of polymer was hydrolyzed with 0.1 M TFA at 95 C for 2.5 h. Upon removal of the TFA by evaporation, an aqueous solution of the product was passed over an Amberlite IR 410/HCO_3^- column as above. This time, the effluent and washings, containing the neutral sugars, were concentrated to dryness, taken up in 0.2 ml of water, and placed on a charcoal (Darco, G60) column (length, 5 cm; area, 1.5 cm^2). The monosaccharides were washed out with water (4 ml/h), and, subsequently, the disaccharide with 5% (vol/vol) aqueous ethanol (44). It was checked for homogeneity by PC (solvent A, R_f relative to galactose = 0.46) and evaporated to dryness (yield: 2 to 3% [wt/wt] from the polymer).

Digestion of oligosaccharides with exo-glycosidases. Portions (2 to 4 mg) of the oligosaccharides were digested with α-glucosidase from yeast (maltase; EC 3.2.1.20; Boehringer, no. 15018), β-glucosidase from sweet almonds (EC 3.2.1.21; Boehringer, no. 15399), or α-galactosidase from green coffee beans (EC 3.2.1.22; Boehringer, no. 15236) under appropriate conditions (11, 17, 19), and using maltose and cellobiose, or melibiose and lactose, respectively, for controlling activity and anomeric specificity. β-Glucuronidase from *Helix pomatia* (EC 3.2.1.31; Boehringer, No. 15472; free of α-glucuronidase activity) was employed in a pH 4.5 acetate buffer (27). The digests were analyzed by PC and GLC of the alditol acetates.

Proton magnetic resonance. The protein magnetic resonance (PMR) spectrum of a 3.5% solution of K29 repeating unit hexasaccharide (P1) (obtained by phage degradation of the polymer as described in the accompanying paper [13]) in absolute deuterium oxide was run at 90 to 95 C (2) using a Varian HR 220 instrument at 220 MHz and the sodium salt of 3-trimethylsilylpropane sulfonic acid as an internal standard.

RESULTS

Homogeneity and composition of E. coli serotype 29 capsular polysaccharide. In agreement with the results of Nhan et al. (30), *E. coli* K29 capsular polysaccharide, as isolated from serogically controlled *E. coli* Bi161/42 [09:K29(A):H^-] by the phenol-water-Cetavlon procedure (21, 22, 30, 43), sedimented homogeneously in the analytical ultracentrifuge after mild alkali treatment, gave but one arc in agar

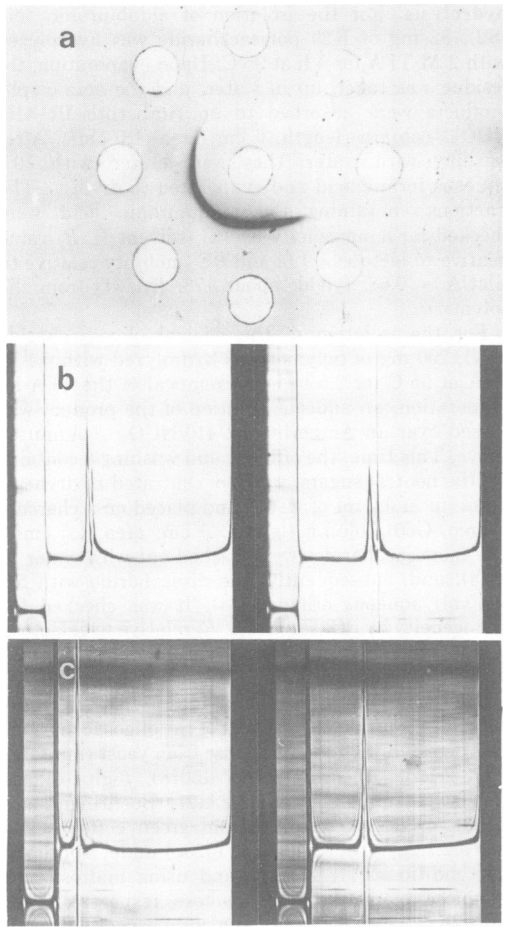


FIG. 1. *Homogeneity of E. coli serotype 29 capsular polysaccharide, as isolated by the phenol-water-Cetavlon technique from E. coli Bi161/42 [09:K29(A):H⁻]. (a) Ouchterlony agar gel double diffusion of native K29 polysaccharide (5, 10, and 20 μg) against an undiluted rabbit serum obtained with formalin-killed E. coli Bi161/42 cells (OK serum). (b) Sedimentation of native K29 polysaccharide (0.4% [wt/vol]; in phosphate-buffered saline) in a Spinco model E analytical ultracentrifuge (An-H-Ti rotor, Schlieren optics) at 60,000 rpm (261,000 × g). Photographs taken 57 and 89 min after start of run; $s^c_{25,PBS}$ = 3.8 × 10⁻¹³ and 4.2 × 10⁻¹³ s. (c) Sedimentation of alkali-treated K29 polysaccharide under the same conditions. Photographs taken 55 and 103 min after start of run; $s^c_{25,PBS}$ = 3.0 × 10⁻¹³ s. The minute contaminant ($s^c_{25,PBS}$ ~ 6 × 10⁻¹³ s) amounts to less than 3% of the major component as judged from the peak areas.*

gel double diffusion against an OK serum obtained with *E. coli* Bi161/42 (Fig. 1) and showed a molar composition approaching Glc:Man:GlcUA:Gal:Pyruvate = 2:2:1:1:1 (found: 2:2.09:1.02:0.93:0.90). No acetyl was detected.

Periodate oxidation. The periodate consumption of serotype 29 capsular polysaccharide is shown in Fig. 2. It can be seen that about 2.4 mol of periodate was taken up quickly, and that the consumption then continued much more slowly, approaching 3 mol per hexasaccharide repeating unit. Upon reduction with sodium borohydride and mild ("Smith") hydrolysis, glycerol could be identified (as its triacetate by GLC on ECNSS-M [retention time: 8.5 min at 110 C]) among the reaction products after 4 h of oxidation, and glycerol and erythritol (retention time of the tetraacetate, 16.5 min at 130 C; peak ratio glycerol triacetate-erythritol tetraacetate found, 1.6:1) could be identified among the Smith degradation products after 7 days of oxidation. One major acidic oligosaccharide (I1) was detected by PE of the Smith degradation products after prolonged oxidation; it was isolated by preparative PE and checked for homogeneity by PC (solvent B) ($[\alpha]_D^{25}$ = +44°).

Methylation analysis of K29 polysaccharide and its derivatives. For selective hydrolysis of pyruvate residues, K29 polysaccharide was subjected to a variety of mild hydrolysis conditions (0.5 N HCl for 24 h at 37 C, or 0.01 N HCl for 1.5 h at 100 C; autohydrolysis of the acidic form for 1 h at 100 C; 0.125% aqueous oxalic acid for 30 min, or for 2 h at 100 C), desalted by passage over a Sephadex G10 column, and analyzed for residual pyruvate content. Treatment with 0.125% oxalic acid for 2 h at 100 C was found to give optimal results, i.e., a polymer (nondialyzable, excluded by Sephadex G50) with only 5.4% of the original pyruvate content. For reduction of carboxyl groups, native and selectively depyruvylated K29 polysaccharide were esterified with ethylene oxide and

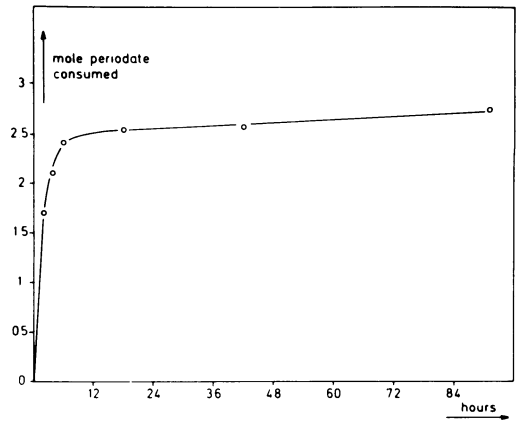


FIG. 2. *Periodate consumption of E. coli serotype 29 capsular polysaccharide (mole per mole of hexasaccharide repeating unit).*

reduced with sodium borohydride (20). Of the GlcUA residues, 80 to 85% was reduced in this manner, as determined by the carbazole-sulfuric acid method. Native, carboxyl-reduced, and depyruvylated and carboxyl-reduced polysaccharide were permethylated and hydrolyzed.

PC (solvent C, *p*-anisidine–trichloroacetic acid spray) of the free methyl-aldoses obtained from native K29 polysaccharide suggested (37; for standards used, see Materials and Methods) the presence of 2,3-di-*O*-methyl-Glc (R_f = 0.26), 2,4,6-tri-*O*-methyl-Gal (R_f = 0.38), 2,4,6-tri-*O*-methyl-Glc (R_f = 0.50), and 3,4,6-tri-*O*-methyl-Man (R_f = 0.58) (the GlcUA derivative does not move under these conditions). The substance with an R_f of 0.26, as well as those with an R_f between 0.50 and 0.60, were isolated by preparative PC and analyzed by GLC as the aldose acetates (3, 9). The whole mixture of methylated sugars was further subjected to GLC-MS of the alditol acetates (5–7, 28). The results, which led to an unequivocal identification in all cases, are given in Fig. 3 and Table 1.

The methylated aldoses obtained from carboxyl-reduced, as well as from depyruvylated and carboxyl-reduced, K29 polysaccharide were analyzed by GLC-MS of the alditol acetates only (Fig. 4 and Table 1). It can be seen that carboxyl reduction resulted in the additional appearance of 2,6-di-*O*-methyl-Glc, and depyruvylation plus carboxyl reduction resulted in the disappearance of most 2,3-di-*O*-methyl-Glc concomitant with the appearance of 2,3,4,6-tetra-*O*-methyl-Glc.

In total, the results of methylation analysis showed that *E. coli* K29 capsular polysaccharide consists of 4,6-substituted Glc, 3-substituted Glc, 2-substituted Man, 3,4-substituted GlcUA, and 3-substituted Gal in a molar ratio approaching 1:1:2:1:1, and that the pyruvate is linked (as a ketal) to positions 4 and 6 of a Glc.

Isolation of oligosaccharides after partial acid hydrolysis. Using different hydrolysis conditions and PC with an acidic irrigant (solvent B) for the detection of acidic oligosaccharides, and with a basic irrigant (solvent A) for the detection of neutral oligosaccharides, optimal conditions for the production of the aldobiuronic acid S2 and the neutral oligosaccharide SN were selected, and these two compounds were isolated by appropriate methods (see Materials and Methods) and checked for homogeneity by PC and PE, or PC only, respectively.

Analysis of oligosaccharides. Oligosaccharides S2, SN, and I1 were analyzed as follows. In agreement with the results of Nhan et al. (30), the aldobiuronic acid S2 yielded galactose as the neutral component upon acid hydrolysis or digestion with β-glucuronidase. After treatment of the neutral oligosaccharide SN with β- (but not with α-) glucosidase, Glc and Man were detected by PC (solvent A), as well as by GLC of the alditol acetates (molar ratio approaching

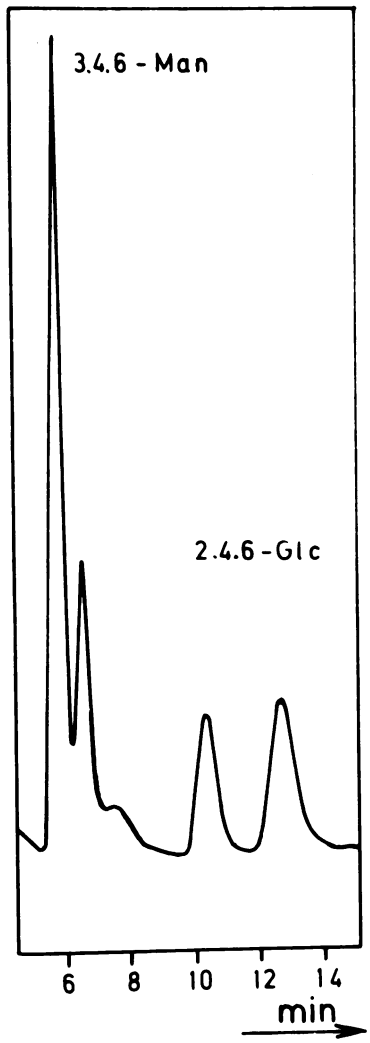


FIG. 3. *Identification and ratio of 3,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-glucose. The mixture of these two compounds, as obtained from permethylated, native K29 polysaccharide, was purified by PC, acetylated (see text), and subjected to GLC on ECNSS-M at 150 C. The aldose acetates (molar ratio = 2:1) showed retention times of 5.8 and 6.6 min (3.4.6-Man), or 10.4 and 12.7 min (2.4.6-Glc); both compounds co-chromatographed with the respective standards and exhibited the same ratio of anomers. Under the same conditions, the aldose acetates of 3.4.6-Glc were found to have retention times of 6.15 and 8.3 min.*

TABLE 1. *Identification and ratios of the O-acetyl-O-methyl-alditols obtained from carboxyl-reduced[a] (I), as well as from depyruvylated and carboxyl-reduced[a] (II) E. coli serotype 29 capsular polysaccharide*

Alditol derivative[b]	T[c]		Primary fragments found (m/e)							Ratio of peak integrals	
	Literature	Found	45	117	161	189	205	233	261	I[a]	II[a]
2.3.4.6-Glc	1.00	1.00[c]	+	+	+		+				0.8
2.4.6-Glc[d]	1.95	1.98[d]	+	+	+	+	+	+		3.2	3.1
3.4.6-Man[d]	1.95										
2.4.6-Gal	2.28	2.31	+	+	+			+		0.9	1.0
2.6-Glc[a]	3.83	3.74	+	+						1.0	1.0
2.3-Glc[e]	5.39	5.39[c]		+	+				+	0.9	0.1

[a] The GlcUA derivative is not obtained with the method used; no 2.6-Glc appeared before carboxyl reduction (conversion of 80 to 85% of the GlcUA to Glc residues).

[b] 2.3.4.6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, etc.

[c] Retention time relative to 2.3.4.6-Glc (T = 1.00) and 2.3-Glc (T = 5.39) on an ECNSS-M column at 170 C (5–7, 28).

[d] 2.4.6-Glc, 3.4.6-Glc, and 3.4.6-Man are not separable as the alditol acetates (see, however, Fig. 3). The mass spectrum of this peak showed primary fragments characteristic for 2.4.6- (m/e = 117), as well as for 3.4.6-substitution (m/e = 189 and 205). The peak integrals of the mixture are given.

[e] Also identified as the aldose acetates; retention time of the anomers: 22 and 26.5 min on ECNSS-M at 150 C (2.3-Man: 41.5 and 56 min). The compounds co-chromatographed with a standard and showed the same ratio of anomers.

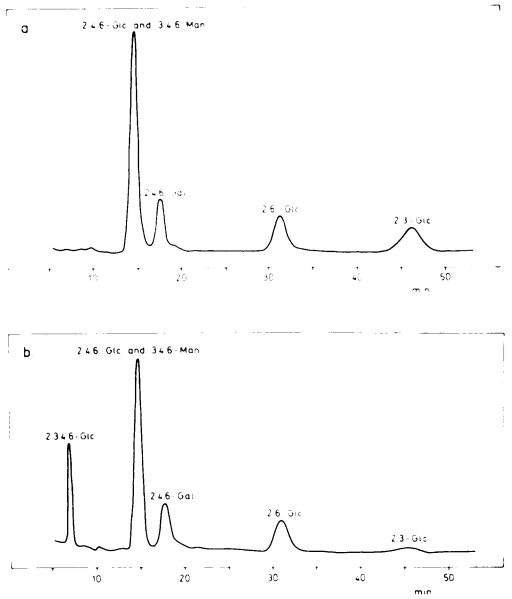


FIG. 4. *GLC of O-acetyl-O-methyl-alditols (on ECNSS-M, isothermal at 145 C) obtained from carboxyl-reduced (a) and depyruvylated and carboxyl-reduced (b) E. coli serotype 29 capsular polysaccharide. No 2.6-Glc is observed before carboxyl-reduction. 2.4.6-Glc = 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol, etc.*

1:1). SN was also permethylated, and the products obtained after hydrolysis were analyzed by PC (solvent C), as well as by GLC of the aldose acetates. 2,3,4,6-Tetra-*O*-methyl-Glc and 3,4,6-tri-*O*-methyl-Man (besides traces of other components) were identified as the major products. After reduction with $NaBH_4$, oligosaccharide I1 was found to consist of Glc:GlcUA:Gal:Glycerol in a molar ratio approaching 1:1:1:1 (found: 1.00:1.18:0.82:0.91). Upon digestion of I1 with β- (but not with α-) glucosidase, Glc was liberated, as evidenced by PC (solvent A) and GLC of the alditol acetate. Sequential treatment of I1 with β-glucosidase, β-glucuronidase, and α-galactosidase led to the formation of free Gal (PC and GLC). At least one α linkage in I1 is also suggested by its optical rotation.

In total, these results are in agreement with the following structural details of the oligosaccharides:

S2 GlcUA $\xrightarrow{\beta}$ Gal

SN Glc-(1 $\xrightarrow{\beta}$ 2)-Man

I1 Glc $\xrightarrow{\beta}$ GlcUA $\xrightarrow{\beta}$ Gal $\xrightarrow{\alpha}$ Glyceraldehyde

Proton magnetic resonance. For PMR, the one repeating unit K29 hexasaccharide (oligosaccharide P1), as obtained by phage 29 digestion of the polymer (see reference 13), was used, because it gave better spectra than the polysaccharide. The material was dissolved in absolute deuterium oxide, and the spectra were run at 90 to 95 C (2). The results are summarized in Table 2.

DISCUSSION

E. coli Bi161/42 [09:K29(A):H$^-$] was isolated by Kauffmann from a case of peritonitis (24). It serves as the serological test strain for the *E. coli*

K29 antigen (25) and is the host of *E. coli* capsule bacteriophage 29 (41). The surface polysaccharides of this organism have been rather extensively studied; its cell wall lipopolysaccharide was found to consist of a core oligosaccharide of the R1 type (35, 36) and an O-specific side chain containing α-(1→2)- and α-(1→3)-linked mannoses (K. Reske, Diplom thesis, University of Freiburg, 1969; K. Reske, personal communication). Its serotype 29 capsular polysaccharide, the receptor of phage 29, has been analyzed by Nhan et al. (30). As yet it is not known whether, and if so how, the capsular glycan is linked to the cell wall constituents underneath. However, the finding (G. Schmidt, personal communication) that, by genetic manipulation, capsules can be transferred to *E. coli* mutants devoid of the outer core and O-specific side-chain regions of the cell wall lipopolysaccharide seems to indicate that it cannot be linked to these structures.

As stated above, an investigation of the products obtained from K29 polysaccharide by depolymerization with phage 29 particles (13, 39, 40) yielded some results, notably upon methylation, which were incompatible with the structure proposed by Nhan et al. (30). This, and the necessity of knowing all anomeric configurations for some further studies (e.g., on the conformation of the polymer and the substrate specificity of the phage glycanase), led us to analyze the primary structure of K29 polysaccharide once more.

The native polymer, as isolated from the bacteria by the phenol-water-Cetavlon procedure (21, 22, 30, 43), gave but one arc in agar gel double diffusion against an *E. coli* Bi161/42 OK serum and sedimented uniformly in the analytical ultracentrifuge after it had been treated with mild alkali (Fig. 1). This behavior is typical for *Enterobacteriaceae* capsular polysaccharides, and it has been repeatedly postulated (e.g., 20, 22, 23, 30) that, in the native material, the single glycan strands may be cross-linked by alkali-labile (possibly uronic acid ester) bonds. In the case of *E. coli* K29 polysaccharide, this view was strengthened by the detection (30; L.B. Nhan, Ph.D. Thesis, University of Freiburg, 1970) of radioactive glucose upon reduction of the native (but not the alkali treated) polymer with $NaBT_4$ and hydrolysis. Other explanations for this phenomenon (e.g., some β-elimination during alkali treatment) are, however, conceivable and necessitate further investigation.

In good agreement with the results of Nhan et al. (30), K29 polysaccharide was found to consist of Glc:Man:GlcUA:Gal:Pyruvate in a molar ratio approaching 2:2:1:1:1, and to carry no or very few *O*-acetyl substituents. This, together with the serological controls of our strain (both the culture, and an OK29 serum were obtained from the World Health Organization International Escherichia Center), and the finding (K. Jann and B. Jann, personal communication) that the polysaccharide analyzed by us, and the K29 serum used by L. B. Nhan, and vice versa, gave identical immunoprecipitation curves (compare [30]), seemed to exclude the possibility that different polymers have been used.

TABLE 2. *Proton magnetic resonance[a] of the E. coli serotype 29 repeating unit hexasaccharide[b]*

δ (ppm)	$J_{1,2}$ (Hz)	Approx ratio of integrals	Proton assignment
1.48		3	CH_3 of pyruvate ketal
4.64	7	1	Axial, anomeric (β linkages)
4.75	7	1.5	
5.30	3.5	0.5	Equatorial, anomeric (α linkages)
5.36	3.5	1	
5.52	3	1	
5.58	3	1	

[a] In deuterium oxide at 90 to 95 C with an internal standard (2, 8).

[b] Obtained by phage-catalyzed depolymerization of K29 polysaccharide (13).

Constituents of the repeating unit. The molar composition of K29 polysaccharide already suggests that it is composed of hexasaccharide repeating units, and this is confirmed by the results of methylation analysis (Table 1), as well as, more directly, by the analysis of the depolymerization products obtained by phage 29 action (see reference 13). Nhan et al. (30) have shown that all constituent sugars belong to the D series (enzymatic determination by glucose oxidase—also, after carboxyl reduction of GlcUA—by galactose oxidase, or via hexokinase, phosphomannose isomerase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase, respectively). In the case of Glc, Gal, and GlcUA, this was now corroborated by hydrolysis with the appropriate exo-glycosidases.

Substitution pattern of the constituents. Our results from methylation analysis disagree with those of Nhan et al. (30). Upon carefully controlled permethylation, the hexasaccharide repeating unit was unequivocally found to consist of 4,6- and 3-substituted Glc, 2-substituted Man, 3,4-substituted GlcUA, and 3-substituted Gal, approaching a molar ratio of 1:1:2:1:1 (see Fig. 3 and 4 and Table 1). The pyruvate was found to be linked (as a ketal) to positions 4 and 6 of a branch terminal Glc. These results are

also in agreement with those of periodate oxidation—consumption of near to 3 mol of periodate (Fig. 2) and production of glycerol upon short (4 h), and, additionally, of erythritol upon prolonged (7 days) oxidation, followed by reduction with sodium borohydride. A comparatively slow and incomplete oxidation of 4,6-*O*-(1-carboxyethylidene)-hexopyranoside branches has been previously observed (H. Thurow et al., Carbohydr. Res., in press), and may result from the increased rigidity of the hexoside chair caused by the ketal substitution (compare Fig. 5).

Sequence of constituents. Our results with oligosaccharides S2 and I1 (oligosaccharides S, obtained by acid ["Säure"] hydrolysis; I, obtained by periodate oxidation; P, obtained by phage degradation [13]) (the terminal glyceraldehyde in the latter must result from a mannose—see above) show that the K29 repeating unit contains a Glc→GlcUA→Gal→Man sequence. Since a branch terminal pyruvylglucose was found by methylation analysis, and since the neutral oligosaccharide SN was identified as Glc→Man, the above tetrasaccharide may constitute the chain sequence, which is substituted by pyruvate→Glc→Man→ branches. The liberation of oligosaccharide SN by mild acid hydrolysis is in agreement with this. The branch sugar is identified by the finding that depyruvylated and carboxyl-reduced serotype 29 polysaccharide contains 3,4-substituted Glc, originating from GlcUA, as the only doubly substituted constituent. Our results do not allow a decision as to which of the two positions of GlcUA is substituted by the branch, or by the chain, respectively; and they do not exclude one alternative, viz., that the branch consists of pyruvyl-glucose only, and that SN is part of the chain also. These details are, however, settled by the finding that phage 29 glycanase depolymerizes the glycan by hydrolysis of Glc-(1→3)-GlcUA linkages (13).

The altered sequence of the K29 hexasaccharide constituents agrees well with the oligosaccharide structures of Nhan et al. (30), all of which (with the exception of S5) can be accomodated in this sequence also.

Anomeric configurations. Besides the signal of the pyruvate ketal methyl protons (2, 8), the PMR spectrum of the K29 repeating unit hexasaccharide gave evidence of approximately 2½ β-[δH(1) < 4.9 ppm], and 3½ α linkages [δH(1) > 4.9 ppm] (Table 2; 2, 33; G. Keilich, personal communication). Since with specific exoglycosidases three β-linkages could be allotted to the two glucoses and the glucuronic acid (see analysis of oligosaccharides S2, SN, and I1), and since the phage 29 enzyme splits the chain β-Glc-(1→3)-GlcUA bonds (13), which accounts for the one sugar present as an anomeric mixture, it follows that the two mannoses and the galactose must be α. This is corroborated by the

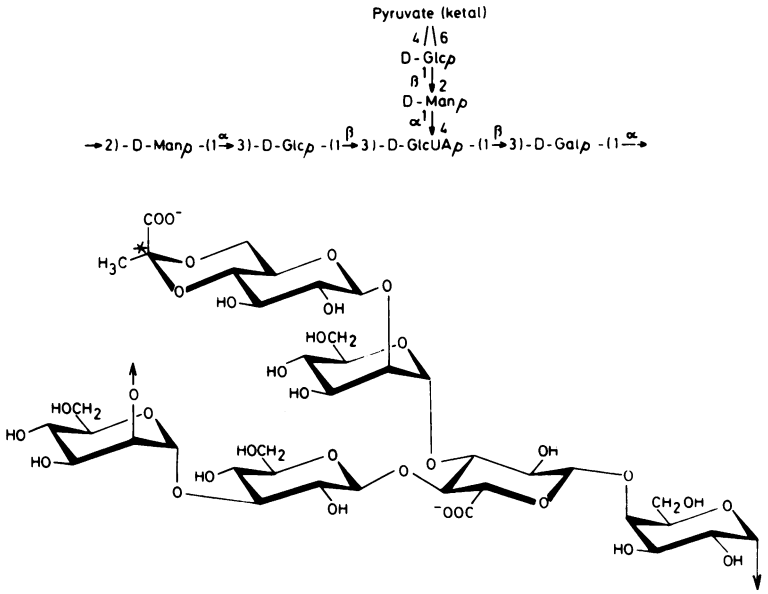


FIG. 5. *Structure of the E. coli serotype 29 capsular polysaccharide (phage 29 receptor) repeating unit. The rotational angles around the glycosidic oxygen bonds, as well as the configuration at C(2) of the pyruvate ketal residue (*) are arbitrary; in analogy to the 4,6-O-(1-carboxyethylidene)-D-glucopyranosyl units in Xanthomonas campestris polysaccharide (15) an axial position of the ketal carboxyl groups was assumed.*

optical rotation of oligosaccharide I1, by the liberation of galactose from it upon sequential digestion with β-glucosidase, β-glucuronidase, and α-galactosidase, and by the large coupling constants ($J_{1,2} \cong 7$) of the two PMR signals at δ = 4.64 and 4.75 ppm, respectively (Table 2), which show that these two axial anomeric protons cannot be contributed by mannoses.

In total, the results—which differ from those of Nhan et al. (30) essentially in methylation analysis only—show that the *E. coli* serotype 29 capsular polysaccharide consists of hexasaccharide repeating units (about 300 per alkali-treated polymer molecule [28]) with the structure given in Fig. 5. Although the angles of rotation around the glycosidic oxygen bonds as well as the configuration at C(2) of the pyruvate ketal residues are not known, the structure, as composed of $^4C_1$ chair monomers, is also depicted—especially to visualize the coplanar arrangement of the two chairs in the 4,6-(1-carboxyethylidene)-D-glucopyranose branch terminals.

ACKNOWLEDGMENTS

We wish to express our gratitude to Hannelore Thoma for excellent technical assistance, to Helga Kochanowski for the ultracentrifugation, and to Ingrid Strohm for the photographic work.

This project was supported by Deutsche Forschungsgemeinschaft and by the National Research Council of Canada.

LITERATURE CITED

1. Avigad, G. 1969. Rapid, sensitive determination of periodate. Carbohydr. Res. **11**:119–123.
2. Bebault, G. M., Y. M. Choy, G. G. S. Dutton, N. Funnell, A. M. Stephen, and M. T. Yang. 1973. Proton magnetic resonance spectroscopy of *Klebsiella* capsular polysaccharides. J. Bacteriol. **113**:1345–1347.
3. Bebault, G. M., G. G. S. Dutton, and R. H. Walker. 1972. Separation by gas-liquid chromatography of tetra-*O*-methylaldohexoses and other sugars as acetates. Carbohydr. Res. **23**:430–432.
4. Bessler, W., F. Fehmel, E. Freund-Mölbert, H. Knüfermann, and S. Stirm. 1975. *Escherichia coli* capsule bacteriophages. IV. Free capsule depolymerase 29. J. Virol. **15**:976–984.
5. Björndal, H., C. G. Hellerqvist, B. Lindberg, and S. Svensson. 1970. Gas-Flüssigkeits-Chromatographie und Massenspektrometrie bei der Methylierungsanalyse von Polysacchariden. Angew. Chemie **82**: 643–674.
6. Björndal, H., B. Lindberg, and S. Svensson. 1967. Gas-liquid chromatography of partially methylated alditols as their acetates. Acta Chem. Scand. **21**:1801–1804.
7. Björndal, H., B. Lindberg, and S. Svensson. 1967. Mass spectrometry of partially methylated alditol acetates. Carbohydr. Res. **5**:433–440.
8. Choy, Y. M., G.G.S. Dutton, A. M. Stephen, and M. T. Yang. 1972. P.M.R. spectroscopic analysis of bacterial polysaccharides containing pyruvic acid. Anal. Lett. **5**:675–681.
9. Choy, Y. M., G. G. S. Dutton, and A. Zanlungo. 1973. The structure of the capsular polysaccharide of *Klebsiella* K-type 24. Can. J. Chem. **51**:1819–1825.
10. Czok, R., and W. Lamprecht. 1970. Pyruvat, phosphoenolpyruvat und D-glyzerat-2-phosphat, p. 1407–1411. *In* H. U. Bergmeyer (ed.), Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim, Bergstrasse, Germany.
11. Dey, P. M., and J. B. Pridham. 1972. Biochemistry of α-galactosidases. Adv. Enzymol. **36**:91–131.
12. Dische, Z. 1962. Colour reactions of hexuronic acids, p. 497–501. *In* R. L. Whistler and M. L. Wolfrom (ed.), Methods in carbohydrate chemistry, vol. 1. Academic Press Inc., New York.
13. Fehmel, F., U. Feige, H. Niemann, and S. Stirm. 1975. *Escherichia coli* capsule bacteriophages. VII. Bacteriophage 29-host capsular polysaccharide interactions. J. Virol. **16**:591–601.
14. Goldstein, I. J., G. W. Hay, B. A. Lewis, and F. Smith. 1965. Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis, p. 361–370. *In* R. L. Whistler and M. L. Wolfrom (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press Inc., New York.
15. Gorin, P. A. J., T. Ishikawa, J. F. T. Spencer, and J. H. Sloneker. 1967. Configuration of the pyruvic acid ketals, 4,6-*O*-linked to D-glucose units, in *Xanthomonas campestris* polysaccharide. Can. J. Chem. **45**:2005–2008.
16. Hakomori, S. 1964. A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) **55**:205–207.
17. Halvorson, H. 1966. α-Glucosidase from yeast, p. 559–562. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
18. Hellerqvist, C. G., B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg. 1968. Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella typhimurium* 395 MS. Carbohydr. Res. **8**:43–55.
19. Hestrin, S., D. S. Feingold, and M. Schramm. 1955. Hexoside hydrolases, p. 231–257. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
20. Hungerer, D., K. Jann, B. Jann, F. Ørskov, and I. Ørskov. 1967. Immunochemistry of K antigens of *Escherichia coli*. 4. The K antigen of *E. coli* 09:K30:H12. Eur. J. Biochem. **2**:115–126.
21. Jann, K., B. Jann, F. Ørskov, and I. Ørskov. 1966. Immunchemische Untersuchungen an K-Antigenen von *Escherichia coli*. III. Isolierung und Untersuchung der chemischen Struktur des sauren Polysaccharids aus *E. coli* 0141:K85(B):H4 (K85-Antigen). Biochem. Z. **346**:368–385.
22. Jann, K., B. Jann, F. Ørskov, I. Ørskov, and O. Westphal. 1965. Immunchemische Untersuchungen an K-Antigenen von *Escherichia coli*. II. Das K-Antigen von *E. coli* 08:K42(A):H⁻. Biochem. Z. **342**:1–22.
23. Jarvis, F. G., M. T. Mesenko, D. G. Martin, and T. D. Perrine. 1967. Physicochemical properties of the Vi antigen before and after mild alkaline hydrolysis. J. Bacteriol. **94**:1406–1410.
24. Kauffmann, F. 1944. Zur Serologie der Coli-Gruppe. Acta Pathol. Microbiol. Scand. **21**:20–45.
25. Kauffmann, F. 1966. The bacteriology of *Enterobacteriaceae*. Munksgaard, Copenhagen.
26. Kickhöfen, B., and O. Westphal. 1952. Papierelektrophorese bei hohen Spannungen zur Trennung von Peptiden. Z. Zaturforsch. **7b**:655–659.
27. Levvy, G. A., and C. A. Marsh. 1960. β-Glucuronidase, p. 397–407. *In* P. D. Boyer, H. Lardy, and K. Myrbäck (ed.), The enzymes, vol. 4. Academic Press Inc., New York.
28. Lönngren, J., and Å. Pilotti. 1971. Gas-liquid chromatog-

raphy of partially methylated alditols as their acetates II. Acta Chem. Scand. **25**:1144–1145.
29. Ludowieg, J., and A. Dorfman. 1960. A micromethod for the colorimetric determination of N-acetyl groups in acid mucopolysaccharides. Biochim. Biophys. Acta **38**:212–218.
30. Nhan, L. B., B. Jann, and K. Jann. 1971. Immunochemistry of K antigens of *Escherichia coli*. The K29 antigen of *E. coli* 09:K29(A):H$^-$. Eur. J. Biochem. **21**:226–234.
31. Ouchterlony, Ö. 1958. Diffusion-in-gel methods for immunological analysis. Progr. Allergy **5**:1–43.
32. Rieger, D., E. Freund-Mölbert, and S. Stirm. 1975. *Escherichia coli* capsule bacteriophages. III. Fragments of bacteriophage 29. J. Virol. **15**:964–975.
33. Rowe, J. J. M., J. Hinton, and K. L. Rowe. 1970. Nuclear magnetic resonance studies on the biochemistry of biopolymers. Chem. Rev. **70**:1–57.
34. Sawardeker, J. S., J. H. Sloneker, and A. Jeanes. 1965. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Chem. **12**:1602–1604.
35. Schmidt, G. 1972. Basalstrukturen von Lipopolysacchariden verschiedener *Enterobacteriaceen*. Genetische und serologische Untersuchungen an R-Mutanten. Zentrabl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Orig. Abt. A **220**:472–476.
36. Schmidt, G., B. Jann, and K. Jann. 1969. Immunochemistry of R lipopolysaccharides of *Escherichia coli*. Different core regions in the lipopolysaccharides of O group 8. Eur. J. Biochem. **10**:501–510.
37. Smith, F., and R. Montgomery. 1959. The chemistry of plant gums and mucilages. Reinhold Publishing Corp., New York.
38. Stirm, S. 1968. *Escherichia coli* capsule bacteriophages. I. Isolation and introductory characterization of five *E. coli* K bacteriophages. J. Virol. **2**:1107–1114.
39. Stirm. S., W. Bessler, F. Fehmel, and E. Freund-Mölbert. 1971. Bacteriophage particles with endo-glycosidase activity. J. Virol. **8**:343–346.
40. Stirm, S., W. Bessler, F. Fehmel, E. Freund-Mölbert, and H. Thurow. 1971. Isolation of spike-formed particles from bacteriophage lysates. Virology **45**:303–308.
41. Stirm, S., and E. Freund-Mölbert. 1971. *Escherichia coli* capsule bacteriophages. II. Morphology. J. Virol. **8**:330–342.
42. Trevelyan, W. E., D. P. Procter, and J. S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.
43. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler and M. L. Wolfrom (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press Inc., New York.
44. Whelan, W. J., J. M. Bailey, and P. J. P. Roberts. 1953. The mechanism of carbohydrase action. Part I. The preparation and properties of maltodextrin substrates. J. Chem. Soc. p. 1293–1298.