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Structural Analysis of Colanic Acid from *Escherichia coli* by using Methylation and Base-Catalysed Fragmentation

COMPARISON WITH POLYSACCHARIDES FROM OTHER BACTERIAL SOURCES

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Essentially the same methanolysis products were obtained after methylation of the slime and capsular polysaccharides from *Escherichia coli* K12 (S53 and S53C sub-strains) and the slime polysaccharides from *E. coli* K12 (S61), *Aerobacter cloacae* N.C.T.C. 5290 and *Salmonella typhimurium* SL1543. These were the methyl glycosides of 2-*O*-methyl-L-fucose, 2,3-di-*O*-methyl-L-fucose, 2,3-di-*O*-methyl-D-glucuronic acid methyl ester, 2,4,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-galactose and the pyruvic acid ketal, 4,6-*O*-(1′-methoxycarbonylethylidene)-2,3-*O*-methyl-D-galactose. All were identified as crystalline derivatives from an *E. coli* polysaccharide. The structure of the ketal was proved by proton-magnetic-resonance and mass spectrometry, and by cleavage to pyruvic acid and 2,3-di-*O*-methyl-D-galactose. All these polysaccharides are therefore regarded as variants on the same fundamental structure for which the name colanic acid is adopted. Although containing the same sugar residues, quite different methanolysis products were obtained after methylation of the extracellular polysaccharide from *Klebsiella aerogenes* (1.2 strain). The hydroxypropyl ester of *E. coli* polysaccharide, when treated with base under anhydrous conditions, underwent β-elimination at the uronate residues with release of a 4,6-*O*-(1′-alkoxycarbonylethylidene)-D-galactose. Together with the identification of 3-*O*-(D-glucopyranosyluronic acid)-D-galactose as a partial hydrolysis product, this establishes the nature of most, if not all, of the side chains as *O*-[4,6-*O*-(1′-carboxyethylidene)-D-galactopyranosyl]-(1→4)-*O*-(D-glucopyranosyluronic acid)-(1→3)-D-galactopyranosyl . . .

The structural chemistry of polysaccharides must be established as a preliminary to any investigation of their conformation, or of the molecular basis of their physical properties or biological function. In these laboratories there is particular interest in polysaccharides that occur naturally as gels or hydrated networks (Rees, 1969): for example, in the problem of how, in molecular-conformational terms, the physical state of an extracellular polysaccharide may change from a coherent capsule to a soluble slime as a result of bacterial mutation. Knowledge of primary structure is also necessary to investigate pathways of polysaccharide biosynthesis and their genetic control and regulation. We here describe the use of methylation analysis and other methods to characterize the primary structures of a group of extracellular polysaccharides as a starting point for wider physical and biological investigations. Some of these polysaccharides correspond to the material that has been named colanic acid (Goebel, 1963).

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MATERIALS AND METHODS

Spectroscopy. N.m.r. spectra were recorded with the Perkin–Elmer R10 (60MHz) and Varian HA-100 (100MHz) spectrometers with tetramethylsilane as internal standard, mass spectra with the AEI MS9 double-focussing mass

spectrometer, u.v. spectra with the Unicam SP.800 recording spectrophotometer, and i.r. spectra with the Perkin–Elmer 237 infrared spectrometer.

Optical rotation measurements. These were made with the Perkin–Elmer 141 automatic polarimeter with 1dm. cells.

Melting points. These were measured on the Kofler hot stage and are uncorrected.

Paper chromatography and electrophoresis. The chromatography solvents were: A, ethyl acetate–pyridine–water (10:4:3, by vol.); B, butan-1-ol–ethanol–water (4:1:5, by vol., upper phase), C, aq. NH_3 (sp.gr. 0·880)–water–butan-2-one (1:17:200, by vol.); D, ethyl acetate–acetic acid–formic acid–water (18:8:3:9, by vol.); E, ethyl acetate–pyridine–water (8:2:1, by vol.); F, ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.); G, butan-1-ol–water–propionic acid (10:7:5, by vol.). The spray reagent was *p*-anisidine hydrochloride in butan-1-ol (Hough, Jones & Wadman, 1950), unless stated otherwise. For electrophoresis, the paper was stretched between two buffer compartments in a closed chamber with no special cooling arrangement; the electrolyte was 0·1M-acetic acid, adjusted with pyridine to pH 6·5, and the potential gradient was about 9v/cm. for periods of up to 2hr.

Gas–liquid chromatography. A Pye Argon Chromatograph (with ^{90}Sr detector) or a Pye series 104 Chromatograph (dual flame ionization detectors) was used with 4ft. or 5ft. glass columns respectively. The liquid phases were: column 1, neopentyl glycol adipate (10% on Gas Chrom P); column 2, polyethylene glycol adipate (15% on Gas Chrom P); column 3, SE-52 silicone oil (3% on Gas Chrom P). The polyester columns were used at 175°, and the silicone column was used at 150°.

Samples were analysed as the equilibrium mixtures of methyl glycosides, which were prepared from the sugars themselves or the polysaccharide methyl ethers (1–10mg.) by treatment with methanolic HCl (2ml.) in a sealed tube, either for 6hr. at 100° or for 16–24hr. at 70°. The reagent was prepared by mixing acetyl chloride (6ml.) and methanol (100ml.). After neutralization with Ag_2CO_3 and filtration, the solution was evaporated to dryness and the residue was dissolved in chloroform (1 drop) for analysis.

Isolation and preliminary characterization of polysaccharides. This is described in the preceding paper (Sutherland, 1969) for *A. cloacae*, *S. typhimurium* and *E. coli* S53. The same methods, including the final deproteinization step with butanol–chloroform, were used to prepare slime polysaccharide from *K. aerogenes* strain and from *E. coli* S61, a mutant of S53 producing a very viscous slime polysaccharide. Capsular polysaccharide from the mutant *E. coli* S53C was isolated by boiling (Dudman & Wilkinson, 1956). For some experiments with *E. coli* S53 polysaccharide, the protein was removed by enzymic digestion. The polysaccharide (30g.) in Sörensen 0·1M-phosphate buffer, pH 7·6 (5l.), was incubated with trypsin (Sigma Chemical Co., St Louis, Mo., U.S.A.) (0·02g.) for 2 days at 35°. The solution was adjusted to pH 6·5 with solid $NaH_2PO_4,2H_2O$ before addition of EDTA (1·2g.), cysteine (2·5g.) and papain (Sigma Chemical Co.) (0·02g.), and further incubation for 2 days at 35°. The solution was dialysed against running tap water (4 days) and passed through an ion-exchange column (IR-120, H^+ form, 300ml. bed vol.) before evaporation to a small volume and freeze-drying (yield, 17·5g.). Lipopolysaccharide was prepared from *E. coli* S53 cells by the phenol–water extraction procedure and purified by ultracentrifugation (Kauffmann, Lüderitz, Stierlin & Westphal, 1960).

Hydrolysis (0·5M-H_2SO_4 at 100° for 16hr.) followed by neutralization with $CaCO_3$, filtration and paper chromatography (solvent A) showed the same mixture of sugars from each extracellular polysaccharide, namely fucose, glucose, galactose and uronic acid. The acid was shown to be glucuronic acid by derivative formation for one polysaccharide and by g.l.c. of a methyl ether methyl glycoside for all except one of the others (see below).

Polysaccharide methyl ethers. Methylation was possible in non-aqueous solvents if the polysaccharides were made soluble by carboxyl esterification. For small-scale preparations, the polysaccharide (0·25g.), in water (500ml.), was passed through an ion-exchange column (Amberlite IR-120, H^+ form) and the solution and washings were evaporated to a small volume (diminished pressure) and freeze-dried. The residue was dispersed in ether (50ml.) and excess of ethereal diazomethane was added at room temperature. After 24hr. the solvent and reagent were removed by evaporation (rotary evaporator) and the residue was dissolved in water and freeze-dried. A solution was made by warming in a mixture of dimethyl sulphoxide (30ml.) and *NN*-dimethylformamide (20ml.), then chilled in ice before adding methyl iodide (10ml.) and $Ba(OH)_2,8H_2O$ (20g.). After being shaken in ice for 2hr., the mixture was allowed to warm to room temperature and shaking was continued for 20hr. Further quantities of reagents were added in the same way on each of the following 2 days, and the solution was finally diluted with water and dialysed against running tap water (3days) before evaporation and freeze-drying. The product was not completely methylated (Found, typically: O·CH_3, 27%), and was therefore treated again under more forcing conditions (Kuhn, Trischmann & Löw, 1955). It was dissolved in *NN*-dimethylformamide (25ml.), and powdered Ag_2O (3g.) was added with methyl iodide (5ml.). The mixture was stirred under reflux (double-surface condenser fitted with drying tube) on a magnetic stirrer–hotplate for 48hr., then diluted with chloroform and filtered. The filtrate was washed with dilute aqueous NaCN (1%), which was then quickly back-washed five times with chloroform; prolonged contact, especially with more concentrated reagent, caused removal of methyl ester. The combined chloroform layers were dried over anhydrous Na_2SO_4, then evaporated to a small volume and poured into excess of light petroleum (b.p. 60–80°). The precipitate was removed on the centrifuge and dried *in vacuo*. If methylation was judged to be incomplete by the presence of O–H stretching bands in the i.r. spectrum, treatment with Ag_2O–methyl iodide was repeated. The yield was typically 0·18g. (Found: O·CH_3, 38·5%).

For the large scale, esterification with ethylene oxide was sometimes used as an alternative to diazomethane. *E. coli* S53 polysaccharide (5g.), in water (3l.), was converted into the free acid with ion-exchange resin as before. The solution was concentrated to about 1l., cooled to 5° before addition of ethylene oxide (50ml.), then securely stoppered and left at room temperature for 4 weeks. After exhaustive dialysis against running tap water to remove by-products, the solution was concentrated and freeze-dried (4·6g.). Part of the product (2·3g.) was dissolved in *N*-methylpyrrolid-2-one (200ml.) with warming. To the cooled solution was added methyl iodide (10ml.) in *N*-methylpyrrolid-2-one (20ml.), with stirring. Then $Ba(OH)_2,8H_2O$ (20g.)

was added at 0°, and the mixture was shaken for 2hr. at 0° and then for 20hr. at room temperature. The same quantities of reagents were added on each of the following 2 days before dilution with water, dialysis against running tap water (3 days), evaporation and freeze-drying (2·0g.) (Found: O·CH_3, 27·3%). Remethylation with Ag_2O (6g.) and methyl iodide (5ml.) in *NN*-dimethylformamide (40ml.), by using the method described above, gave an improved product (1·6g.) (Found: O·CH_3, 33·8%). Extensive saponification must have occurred at some stage, perhaps as a result of slower handling during the large-scale treatment with cyanide, because passage of a chloroform solution through ion-exchange resin (Amberlyst 15, H^+ form), followed by evaporation and treatment with ethereal diazomethane, led to an increased methoxyl content (Found: O·CH_3, 40·8%) and a pronounced decrease in intensity of an i.r. band at 1665cm.$^{-1}$, which could be attributed to carboxylate groups. The methoxyl content could not be increased further by treatment with Ag_2O–methyl iodide or diazomethane.

EXPERIMENTAL AND RESULTS

Identification of the hydrolysis products of E. coli *S53 polysaccharide*

Hydrolysis and preliminary separation on an ion-exchange column. Polysaccharide (2g.) was hydrolysed on a boiling-water bath with M-sulphuric acid (400ml.) for 12hr. The solution was neutralized with calcium carbonate, cooled in ice–water, then filtered and concentrated in a rotary evaporator. The syrup, in water (25ml.), was passed through Amberlite IR-120 (H^+ form) resin (bed vol. 100ml., column diam. 18mm.), which was then washed with water until the effluent gave a negative colour reaction (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) for carbohydrate (6l.). Carbohydrate was not displaced when the column was subsequently washed with 2M-hydrochloric acid.

The washings that contained carbohydrate were combined and evaporated to 25ml., then passed through an ion-exchange resin (Amberlite IRA-400, acetate form; bed vol. 100ml., diam. 18mm.), and washed through with water. This fraction contained neutral sugars only (see below) and was evaporated to a syrup under diminished pressure. Uronic acids were displaced with M-formic acid (500ml.) and evaporated to a syrup, which was stored over potassium hydroxide pellets.

Separation of neutral sugars. The syrup was applied to a cellulose column (30cm. × 2·5cm. diam.) and eluted with ethyl acetate–pyridine–water (16:4:1, by vol.), with fractions (20ml.) being collected automatically and monitored by paper chromatography. Three larger fractions, each containing a single sugar, were obtained by combining these solutions and evaporation to dryness. Fraction 1 (0·59g.) contained L-fucose only, characterized by conversion into the toluene-*p*-sulphonylhydrazone (Easterby, Hough & Jones, 1951), m.p. and mixed m.p. 170°. Fraction 2 (0·30g.) was D-glucose, which gave (Bell, 1947) the 1,3:5,6-di-*O*-isopropylidene derivative, m.p. and mixed m.p. 109–110°, after recrystallization from ether–light petroleum (b.p. 40–60°). Fraction 3 (0·53g.) was D-galactose, characterized by conversion (Hirst, Jones & Woods, 1947) into the *N*-methyl-*N*-phenylhydrazone, m.p. and mixed m.p. 185°.

Separation of acidic sugars. This was done in the same way as for the neutral sugars, except that the eluting solvent was ethyl acetate–acetic acid–formic acid–water (18:3:1:2, by vol.). Fraction 1 from this column (0·14g.) was D-glucurone, which gave (Owen, Peat & Jones, 1941) methyl β-D-glucofuranuronoside (6→3)-lactone, m.p. 139·5°, mixed m.p. 138°. Fraction 2 (0·15g.) was D-glucuronic acid, which gave the same derivative, m.p. and mixed m.p. 138°. Fraction 3 (0·08g.) was the aldobiuronic acid described below.

3-O-(D-*Glucopyranosyluronic acid*)-D-*galactose.* Further quantities of this were prepared in the same way as previously, but under milder hydrolysis conditions (0·5M-sulphuric acid for 12hr.). It had $[\alpha]_D$ +38° (*c* 2·0 in water) and the apparent degree of polymerization was 2·2 as measured by the method of Peat, Whelan & Roberts (1956) adapted for the phenol–sulphuric acid reagents (Dubois *et al.* 1956). Hydrolysis (M-sulphuric acid at 100° for 16hr.), followed by neutralization and paper chromatography in the usual way (solvent A), gave galactose and uronic acid, suggesting that the substance was a glucuronosylgalactose, probably identical with the 3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose that was reported (Rodén & Markovitz, 1966) at the time our experiments were in progress. To establish the position of linkage, the hydrolysis products were examined after methylation and carboxyl reduction. The acid (0·09g.), in cold 10% (w/v) sodium hydrogen carbonate solution (50ml.), was stirred while 30% (w/v) sodium hydroxide (30ml.) and methyl sulphate (10ml.) were added simultaneously and dropwise over 3 days, with the solution kept in an ice bath for the first 2hr. After acidification to pH2 with 3M-sulphuric acid, the partly methylated product was extracted with chloroform and dried over sodium sulphate. The residue after evaporation of chloroform (0·085g.) was dissolved in tetrahydrofuran (10ml.) and treated with excess of lithium aluminium hydride for 16hr. at room temperature and then 4hr. under reflux. Excess of hydride was destroyed with ethyl acetate, and the minimum of water was added to precipitate lithium aluminate. After filtration and evaporation, the residue was dried over phosphoric oxide and dissolved in *NN*-dimethylformamide

(10ml.) containing methyl iodide (1ml.). This solution was cooled to 0° before addition of silver oxide (0·50g.) and shaking, first in ice (2hr.) and then at room temperature (3 days). Solvent was removed in the rotary evaporator, and the product was obtained by extraction of the residue with chloroform (0·070g.).

Methanolysis and g.l.c. showed only the glycosides of 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose when run on columns 1 and 2. The presumed disaccharide derivative was hydrolysed by heating in aqueous 45% (v/v) formic acid for 10hr. at 100°, then the acid was removed by evaporation, first in the rotary evaporator and then in a desiccator over potassium hydroxide pellets. Paper chromatography with solvents B and C showed two spots only, corresponding to 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose. After separation on paper sheets (solvent B), the galactose derivative crystallized (m.p. 84°, mixed m.p. 85°).

Methylation analysis of E. coli *polysaccharide*

Hydrolysis of the methylated polysaccharide and separation of the products. Methylated polysaccharide (1·5g.), in aqueous 45% (v/v) formic acid, was heated at 100° for 24hr. before removal of the acid by evaporation, first in the rotary evaporator and then in a desiccator over potassium hydroxide pellets. The syrupy product was applied to a cellulose column (28cm. × 5cm. diam.), which was then eluted with light petroleum (b.p. 100–120°)–butanol (3:1, v/v). Fractions (25ml.) were collected auto-

Table 1. *G.l.c. of methylated sugars as their equilibrium mixtures of methyl glycosides*

Experimental conditions are given in the text. Retention times are relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. Each peak is designated *s*, *m*, *w* or *sh*, to indicate respectively whether it is of strong, medium or weak intensity, or occurs as a shoulder on another peak.

Source	Parent sugar	Relative retention time			
		Column 1		Column 2	
Reference sample	2,3,4,6-Tetra-*O*-methylglucose	1·00*m*	1·38*s*	1·00*m*	1·43*s*
	2,4,6-Tri-*O*-methylglucose	2·56*m*	3·64*s*	3·28*m*	4·82*s*
	2,3,6-Tri-*O*-methylglucose	2·78*m*	3·64*s*	3·56*m*	4·81*s*
	2,4-Di-*O*-methylglucose	5·88*m*	7·85*s*	10·1*m*	13·7*s*
	4,6-Di-*O*-methylglucose	5·92*s*	7·30*m*		
	2,3-Di-*O*-methylglucose	7·20*m*	9·20*s*		
	2,3,4,6-Tetra-*O*-methylgalactose	1·70		1·89	
	2,4,6-Tri-*O*-methylgalactose	3·08*m*	3·50*s*	4·08*m*	4·69*s*
	2,3,6-Tri-*O*-methylgalactose	2·52*s*	3·18*w*	3·11*s*	3·72*w*
		3·50*m*		4·11*w*	4·50*m*
	2,3-Di-*O*-methylgalactose	6·56*s*	9·80*m*		
	2,6-Di-*O*-methylgalactose	5·21*w*	6·08*s*		
		6·98*w*	7·77*m*		
	4,6-Di-*O*-methylgalactose	5·15*s*	8·40*m*	8·28*s*	14·60*m*
	2,4-Di-*O*-methylgalactose	9·51*m*	10·70*s*	8·35*m*	8·95*w*
				10·60*w*	11·43*s*
	2,3,4-Tri-*O*-methylfucose	0·72		0·85	
	2,3-Di-*O*-methylfucose	0·90*s*	1·15*sh*	1·05*s*	1·44*sh*
		1·18*m*	1·24*w*	1·46*m*	1·50*w*
	2-*O*-Methylfucose	2·58*w*	2·86*s*	4·05*s*	4·70*m*
		2·92*m*	3·80*w*	5·05*w*	
	2,3,4-Tri-*O*-methylglucuronic acid	2·26			
	2,3-Di-*O*-methylglucuronic acid	5·43*m*	6·00*m*	8·05*s*	8·84*w*
		6·81*s*	8·50*w*	10·70*m*	
Cleavage products from methylated polysaccharide from *E. coli* S53	Fraction 1	13·50		17·50	
	Fraction 2	0·90*s*	1·16*sh*	1·05*s*	1·44*sh*
		1·19*m*	1·24*w*	1·47*m*	1·50*w*
	Fraction 3 First subfraction	2·55*m*	3·62*s*	3·28*m*	4·81*s*
	Second subfraction	3·06*m*	3·50*s*	4·08*m*	4·69*s*
	Fraction 4	2·58*w*	2·86*s*	4·00*s*	4·68*m*
		2·92*m*			
	Fraction 5	1·10*w*	8·40*s*		
	Fraction 6	5·43*m*	6·78*s*	8·05*s*	10·66*m*

matically and combined into larger fractions on the basis of analysis by paper chromatography. After the first component had emerged, the solvent mixture was changed to the proportions 1:1 (v/v) and then to 1:3 (v/v). Finally, the column was eluted with butanol half-saturated with water.

Fraction 1 (0·025g.) did not correspond in its chromatographic properties to any known simple glycoside; the complete investigation of this material is described below, where it is shown to be a pyruvic acid ketal.

Fraction 2 (0·125g.) contained a major component that corresponded, on gas chromatograms (Table 1) and paper chromatograms, to 2,3-di-*O*-methylfucose. It was slightly contaminated with the material present in fraction 1 and was therefore purified on paper sheets (solvent B) before conversion into the crystalline methyl α-pyranoside, m.p. 48–50° [Conchie & Percival (1950) give m.p. 49–51°].

Fraction 3 was a mixture of two sugars that corresponded, on gas chromatograms (Table 1) and paper chromatograms, to 2,4,6-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose. Separation was achieved on a small cellulose column by elution with aq. ammonia (sp.gr. 0·880)–water–butan-2-one (1:34:400, by vol.). The glucose derivative (0·275g.) was recrystallized from ether and had m.p. and mixed m.p. 120°. The galactose derivative (0·420g.) was recrystallized from light petroleum (b.p. 40–60°)–ether, and had m.p. and mixed m.p. 100°.

Fraction 4 (0·140g.) crystallized directly, m.p. 147–150°, corresponding (Aspinall, Jamieson & Wilkinson, 1956) to 2-*O*-methylfucose. This identity is confirmed by the behaviour on gas chromatograms (Table 1) and paper chromatograms.

Fraction 5 (0·023g.) behaved as a di-*O*-methylhexose on paper chromatograms, and was evidently a glucose derivative because treatment with silver oxide and methyl iodide in *NN*-dimethylformamide (conditions as above) gave 2,3,4,6-tetra-*O*-methylglucosides, identified by g.l.c. It was probably 2,6-di-*O*-methylglucose because it was different from the 2,3- and 4,6-dimethyl ethers (Table 1) and its glycosides could not be detected by g.l.c. after they had been kept for 24hr. in 0·1M-sodium metaperiodate.

Fraction 6 (0·116g.) moved as a single spot on paper chromatograms in several solvent systems, and was electrophoretically mobile in pyridine–acetic acid buffer, suggesting a uronic acid derivative. It was identified by g.l.c. as 2,3-di-*O*-methylglucuronic acid (Table 1), this conclusion being confirmed by conversion into the *p*-nitrobenzoate of the methyl ester methyl glycoside, which, after four recrystallizations from ethanol, had m.p. 155° [Smith (1940) reported m.p. 157°].

Pyruvic ketals from E. coli *polysaccharides*

Optimum conditions for cleavage of the methylated polysaccharide. Low and variable amounts of the ketal were obtained by acid hydrolysis (see above), but the yields were larger when the polysaccharide methyl ether was fragmented by methanolysis. Methylated slime polysaccharide from *E. coli* K12 S53 (0·100g.) was suspended in methanolic hydrogen chloride (20ml., prepared by prior mixing of acetyl chloride, methanol and 2,2-dimethoxypropane in the proportions 1:20:1, by vol.), and mixed with an equilibrium mixture of methyl 4,6-di-*O*-methyl-D-galactosides (2–5mg.) as the internal standard for quantitative g.l.c. Samples were removed after refluxing for various periods of time and, after neutralization of each with silver carbonate and filtration, the release of the ketal residue was estimated from relative peak heights on g.l.c. (column 2). The results (Table 2) suggested that the best yield was obtained after 4hr.

Isolation of methyl 4,6-O-(1′-*methoxycarbonylethylidene*)-2,3-*di*-O-*methyl*-α-*and*β-D-*galactopyranosides.* Methylated slime polysaccharide from *E. coli* K12 S53 (3g.), suspended in methanolic hydrogen chloride (300ml., prepared as on the small scale), was heated under reflux for 4hr. The solution was cooled, neutralized with silver carbonate, filtered and evaporated to 25ml. for application to a silica-gel column (36cm. × 4cm. diam.) previously equilibrated with ether. Elution with ether (2l.) was followed by ether–benzene (3:1, v/v) (2l.) and then chloroform (2l.), with fractions (50ml.) collected automatically and monitored by g.l.c. on column 3. This column resolved the ketal peak into two components, presumably the anomeric glycosides, having retention times, relative to methyl tetra-*O*-methyl-β-glucoside, 4·05 and 4·25. The fractions that contained ketal glycosides were combined and

Table 2. *Production of methyl* 4,6-O-(1′-*methoxycarbonylethylidene*)-2,3-*di*-O-*methyl*-αβ-*galacto*-*pyranoside by methanolysis of the slime polysaccharide from* E. coli *K*12 *S*53 *after methylation*

The preparation of samples is described in the text. The Pye Argon Chromatograph was used with column (2).

	Peak heights measured from chart (cm.)		
Methanolysis period (hr.)	Ketal	4,6-Di-*O*-methyl-galactoside	Ratio
1	1·80	7·40	0·240
2	3·20	8·70	0·370
4	3·60	9·60	0·375
8	2·85	7·85	0·363
24	3·20	9·00	0·355

evaporated to dryness (0·32g.). Small amounts of 2,3-di-*O*-methylfucoside contaminants were present and the mixture was therefore methylated again with silver oxide and methyl iodide in *NN*-dimethylformamide and rechromatographed on silica gel in the same way, when the 2,3,4-tri-*O*-methyfucosides were easily removed. The ketal glycosides were themselves partly resolved and were designated B (the faster-moving on g.l.c.; 0·050g.) and A (0·180g.). A mixed fraction (0·030g.) was also obtained. The fraction B crystallized, and was recrystallized from carbon disulphide, m.p. 107–108°, $[\alpha]_D$ −1·00° (*c* 0·9 in chloroform) (Found: C, 50·7; H, 7·3; $C_{13}H_{22}O_8$ requires C, 51·0; H, 7·2%). Fraction A remained a syrup, $[\alpha]_D$ +81·5° (*c* 0·4 in chloroform).

Similar treatment of the slime polysaccharide from *E. coli* K12 S61 gave the same two compounds; the crystalline product had m.p. 108–110° and did not depress the m.p. of compound B.

Confirmation that compounds A and B were anomeric glycosides was that each was converted into the same equilibrium mixture, as shown by g.l.c. on column 3, when treated with methanolic hydrogen chloride (3% at 100° for 5hr.). The optical rotations suggest that the α-configuration may be assigned to compound A and the β-configuration to compound B, if the parent sugar is in the D-series. These conclusions are borne out by the spectroscopic results described below.

Structures of the glycoside ketals by spectroscopic methods. Hydroxyl absorption was not observed in the i.r. spectrum of either compound A or compound B when measured with 2% solutions in chloroform and 0·5mm. liquid cells; each showed a strong carbonyl absorption at 1743cm.$^{-1}$ to indicate an ester function, or possibly a cyclic ketone.

N.m.r. spectra were recorded for both compounds A and B in deuterochloroform, benzene, carbon disulphide and carbon tetrachloride. The spectra for compounds A and B were similar and consistent with the conclusion that they differed only in configuration at C-1. The best resolution was for compound B in benzene (Fig. 1), and detailed information about chemical shifts and coupling constants is given for this spectrum as an example. Interpretation is based on the assumption that the compound contains 22 protons, to fit the molecular formula suggested by mass spectrometry (see below):

(i) A singlet at 8·42τ, corresponding to three protons, was assigned to a *C*-methyl group, which must be attached to a quaternary carbon atom.

(ii) Two singlets at 6·62τ (six protons) and 6·65τ (3 protons) were assigned to three *O*-methyl groups, presumably representing two methyl ethers and a glycosidic methyl group.

(iii) A singlet corresponding to three protons at 6·40τ, was shown to represent a methyl ester by

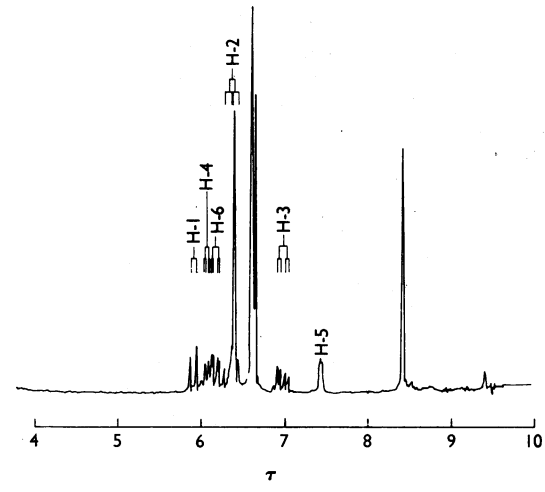


Fig. 1. N.m.r. spectrum, at 100MHz in benzene solution, of methyl 4,6-*O*-(1′-methoxycarbonylethylidene)-2,3-di-*O*-methyl-β-D-galactopyranoside. Assignments of the sugar ring protons are labelled; for assignment of the methyl group singlets see the text.

the following experiments. To the compound A (0·100g.), in redistilled tetrahydrofuran (20ml.), was added lithium aluminium hydride (0·100g.), and the mixture was kept at room temperature for 8hr. and then boiled under reflux for 4hr. After the destruction of excess of reagent with ethyl acetate, and precipitation of inorganic salts by the minimum of water, the syrupy product after filtration and evaporation was dissolved in chloroform, washed with water and dried over sodium sulphate. The n.m.r. spectrum showed no peak at 6·40τ, but a characteristically broad singlet at 7·30τ corresponding to a hydroxyl proton was present. The i.r. spectrum showed the disappearance of the carbonyl band at 1748cm.$^{-1}$ with appearance of OH absorption at 3400cm.$^{-1}$. All this points to the conversion $-CO\cdot O\cdot CH_3$ into $-CH_2\cdot OH$ by lithium aluminium hydride. As further confirmation, the syrupy reduction product was acetylated in acetic anhydride–pyridine (2:3, v/v) (15ml.) at 100° for 2hr. After removal of the reagents by evaporation, the acetate was dissolved in chloroform and washed with aqueous copper sulphate, aqueous sodium carbonate and water, and then dried over sodium sulphate. N.m.r. showed a new singlet at 7·90τ, corresponding to one *O*-acetyl group, with disappearance of the signal at 7·30τ. The i.r. spectrum likewise showed replacement of hydroxyl absorption (3400cm.$^{-1}$) by ester (1725cm.$^{-1}$).

(iv) A system of seven protons, although forming a rather complex pattern, could be individually assigned by spin decoupling to the protons of

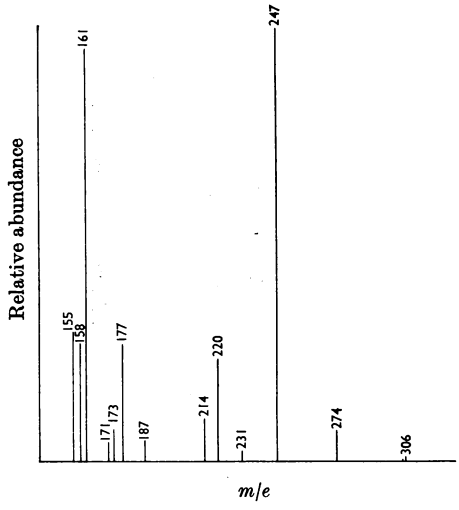


Fig. 2. Mass spectrum of methyl 4,6-*O*-(1′-methoxycarbonylethylidene)-2,3-di-*O*-methyl-β-D-galactopyranoside. For the interpretation see Scheme 1.

the sugar ring (Fig. 1). The conclusions were checked, as far as possible, by using the spectra recorded in other solvents. (*a*) The H-1 signal was recognized by its appearance at the lowest field, 5·90τ. It was a doublet with $J_{1,2}$ 7·5Hz, consistent only with axial protons at H-1 and H-2 on a pyranoside ring. (*b*) The H-2 signal, recognized by being coupled to H-1, was a quartet at 6·36τ with $J_{2,3}$ 9·0Hz, showing that H-3 is also axial. (*c*) H-3, at 6·98τ, showed $J_{3,4}$ 3·5Hz and thus that H-4 is equatorial. (*d*) H-4, at 6·07τ, showed $J_{4,5}$ 1Hz, which gives no basis for assignment of configuration at C-5. (*e*) H-5 was a complex multiplet at 7·43τ. (*f*) The two H-6 protons, appearing as a multiplet at 6·18τ, were coupled with each other, with *J* 7·5–8·0Hz. This means that bond rotation about C-5–C-6 must be prevented in some way.

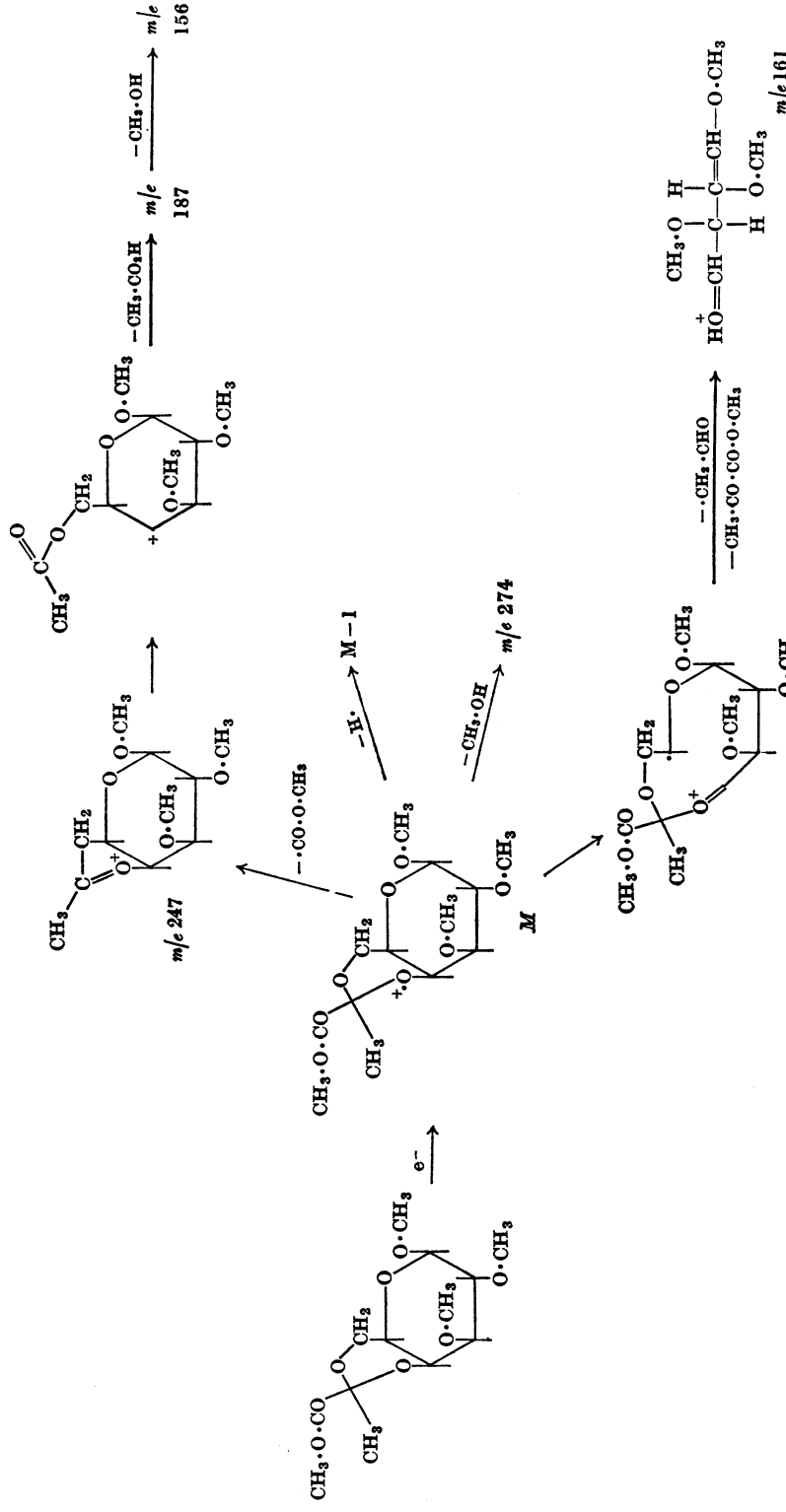
The mass spectra of compounds A and B were identical (Fig. 2), as would be expected for anomers. The peaks corresponding to highest mass were *m/e* 306·13412 ($C_{13}H_{22}O_8$) and 305·12447 ($C_{13}H_{21}O_8$), which could be assigned to *M* (parent ion) and *M* − 1 respectively. The abundant peaks in the high-mass region could be explained on the basis of the structure that emerged from n.m.r. analysis, by analogy with pathways already proposed (Kotchetkov & Chizhov, 1966) for isopropylidene ketals (Scheme 1). The step *m/e* 247 → 187 was confirmed by a metastable peak. The breakdown is evidently dominated by the ketal ring.

The combined spectroscopic evidence is consistent only with the structural formula (I) for compound B, and the corresponding α-glycoside for compound A. (The necessity for the 4,6-ring fixes the stereochemistry at C-5, which was the only ambiguity from the n.m.r. spectrum.)

Confirmation of the structure of compound B by chemical methods. The crystalline material (10mg.), in dry dichloromethane (2ml.), was cooled in acetone–solid carbon dioxide before addition of boron trichloride (1g.) that had been precooled in the same way (Bonner, Bourne & McNally, 1960). After 30min. in this bath, the mixture was allowed to warm to room temperature and kept under anhydrous conditions for 16hr. before evaporation to dryness and repeated distillation of methanol from the residue. Analysis by paper chromatography (solvent A) showed galactose as the only product.

A further sample of compound A (35mg.) was heated at 100° in 0·5M-sulphuric acid (5ml.) for 5hr. The solution was cooled before neutralization with aq. 0·15M-barium hydroxide, filtration and evaporation to small volume; paper chromatography (solvent G) with the *o*-phenylenediamine–trichloroacetic acid spray (Wieland & Fischer, 1949) showed a spot with the same mobility and colour as barium pyruvate. The solution was treated with cation-exchange resin (Amberlite IR-120, H^+ form) and extracted with ether. Evaporation of the ether gave pyruvic acid, which, with redistilled phenylhydrazine in ethanol, gave the phenylhydrazone, m.p. 183–185°, mixed m.p. 185–187°. Analysis of the aqueous layer by paper chromatography (solvent C) showed a spot with the same mobility as 2,3-di-*O*-methylgalactose. Evaporation to dryness followed by treatment under reflux with ethanol (5ml.) containing redistilled aniline (0·05ml.) gave 2,3-di-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 145–147°, mixed m.p. 149–151°.

Isolation of the ketal without methylation. The polysaccharide from *E. coli* S53 was esterified with propylene oxide by using an equivalent procedure to that described in the Materials and Methods section with ethylene oxide. The hydroxypropyl ester (1g.) was shaken at room temperature with methanol (40ml.) containing 2,2-dimethoxypropane (5ml.) to remove water by chemical reaction (McCleary, Rees, Samuel & Steele, 1967) before mixing with dry methanolic 0·04M-sodium methoxide (40ml.) and shaking for a further 24hr. The yellow solution, which showed strongly the absorption at 233nm. in the ultraviolet that is characteristic of αβ-unsaturated esters, was neutralized with M-hydrochloric acid and evaporated to dryness. The residue was extracted several times with water, leaving some insoluble material ('fraction C', 0·60g.). The combined solutions were evaporated to 5ml. before the addition of acetone (50ml.) to precipitate polymeric material ('fraction D',

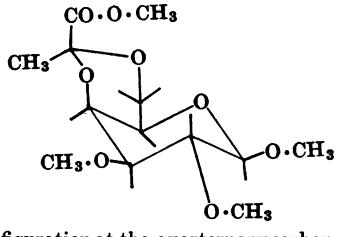


Scheme 1. Fragmentation of methyl 4,6-*O*-(1′-methoxycarbonylethylidene)-2,3-di-*O*-methyl-β-D-galactopyranoside in the mass spectrometer.

0·35g.). Evaporation of the aqueous acetone gave a residue ('fraction E', 0·12g.).

Fraction D was evidently polymeric because it gave no detectable sugars when examined by paper chromatography (solvent E), and the degree of polymerization, as determined by the method of Peat *et al.* (1956), exceeded 10. Methylation followed by methanolysis and g.l.c. showed a very similar pattern of glycosides to that obtained directly from the *E. coli* polysaccharide. Similar analysis of fraction C, however, showed that the ketal peak had diminished to a relative concentration of about one-half. Comparison of fraction C with intact colanic acid, by hydrolysis with aqueous formic acid followed by paper chromatography in the usual way, showed that substantially less glucuronic acid residues were present in fraction C.

Fraction E, in aqueous solution, was passed through mixed Amberlite IR-120 (H^+ form) and IR-45 (free base form) resins and then reisolated by evaporation to dryness (0·035g.). It appeared to contain monosaccharide derivatives only, because there was no reaction with the phenol–sulphuric acid reagents (Dubois *et al.* 1956) after reduction with borohydride (compare Peat *et al.* 1956). Paper chromatography showed a single fast-moving spot with R_{Glc} values 2·05 (solvent E) and 1·9 (solvent F). The corresponding values for fucose were 1·7 and 1·6. It gave a brown colour with *p*-anisidine hydrochloride spray, and was electrophoretically immobile in pyridine–acetic acid buffer. Methylation with silver oxide and methyl iodide in *NN*-dimethylformamide at 0° for the first 2hr. and then at room temperature, followed by the usual preparation and by methanolysis, gave a product that was identical by g.l.c. with methyl 4,6-*O*-(1′-methoxycarbonylethylidene)-2,3-di-*O*-methyl-αβ-galactopyranoside.



(I)* (the configuration at the quarternary carbon is assumed)

Comparison of colanic acid and other polysaccharides from various sources

The capsular and slime polysaccharides from *E. coli* K12 S53, and the slime polysaccharides from *E. coli* K12 S61 and *A. cloacae* N.C.T.C. 5920, all gave identical gas chromatograms after methylation and methanolysis. An example is shown in Fig. 3. The slime polysaccharide of *S. typhimurium* SL 1543 was also similar but showed small amounts of 2,3,4,6-tetra-*O*-methylgalactosides and a correspondingly smaller ketal peak. It was estimated from the peak areas that, relative to the proportion of 2,4,6-tri-*O*-methylgalactosides, the methanolysis products of this polysaccharide contained about 15% less ketal than the others.

No attempt was made to identify the methylation and methanolysis products from the *K. aerogenes* polysaccharides or the *E. coli* K12 S53 lipopolysaccharide, other than to show that they were very different from the five mentioned above, and, in particular, that the ketal derivative was absent.

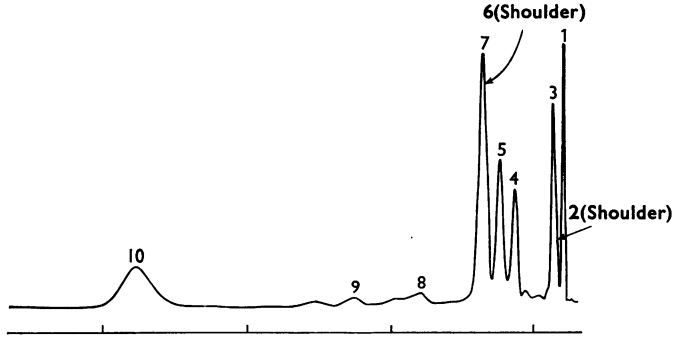


Fig. 3. Methanolysis products of the methylated polysaccharide from *E. coli* S53, chromatographed on polyethylene glycol adipate at 175° (Pye 104 Chromatograph). The derivatives present are: 2,3-di-*O*-methylfucosides (peaks 1, 2 and 3); 2,4,6-tri-*O*-methylglucosides (peaks 4 and 7); 2,4,6-tri-*O*-methylgalactosides (peaks 5 and 6); 2-*O*-methylfucosides (peaks 5 and 6); 2,3-di-*O*-methylglucuronoside methyl esters (peaks 8 and 9); 4,6-*O*-(1′-methoxycarbonylethylidene)-2,3-di-*O*-methylgalactosides (peak 10).

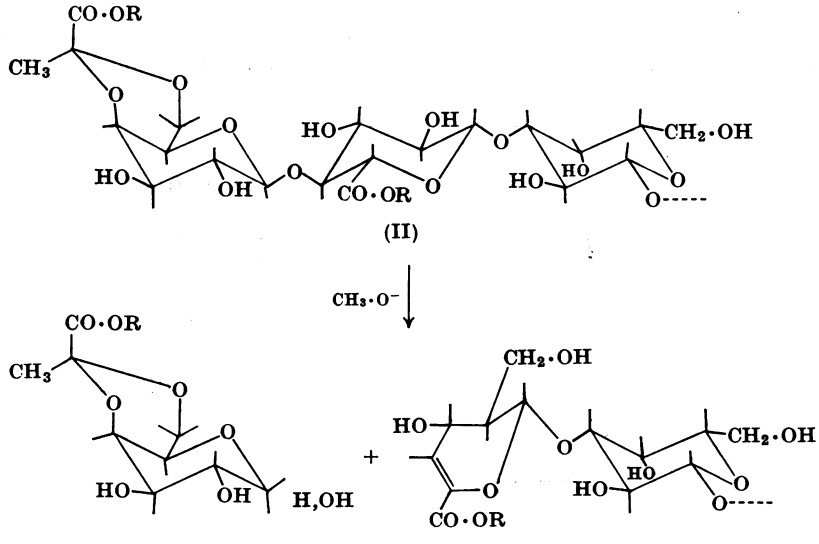
The only similarity was that the polysaccharide from *K. aerogenes* 1.2 gave 2,3-di-*O*-methylfucosides.

DISCUSSION

Methylation analysis has been used to characterize the polysaccharide structures reported in this paper, together with partial fragmentation by β-elimination. Both methods involve conditions that are sufficiently basic to remove any *O*-acyl groups and no information can therefore be obtained about the presence or distribution of these substituents, although they are known to occur in extracellular polysaccharides (Sutherland, 1967), including some of those examined here (Sutherland, 1969). Similarly, we cannot discuss whether either type of acidic sugar residue is carboxyl-esterified in the native state.

The most detailed investigations were of the slime polysaccharide from *E. coli* K12 S53, which contains residues of galactose, glucose, fucose and glucuronic acid in the approximate molar proportions 2:1:2:1 (Sutherland, 1969). Each sugar in the molar proportion of 2 gave two different methyl ethers after methylation and hydrolysis, whereas the others each gave a single methyl ether. (The di-*O*-methylglucose is disregarded since it probably results from undermethylation.) The isolation of 2-*O*-methyl-L-fucose suggested a branched structure for the polysaccharide, which was confirmed by the identification of galactose derivatives in substantial proportions that could have arisen only from chain ends, namely the methyl ester methyl ether methyl glycosides of 4,6-*O*-(1′-carboxyethylidene)-D-galactose. The same pyruvic acid ketal occurs in agaropectin (Hirase, 1957) and in *Corynebacterium insidiosum* polysaccharide (Gorin & Spencer, 1964), and the corresponding glucose ketal is an end group in *Xanthomonas campestris* polysaccharide (Sloneker & Orentas, 1962; Gorin, Ishikawa, Spencer & Sloneker, 1967). The configuration at the quaternary carbon atom usually corresponds to an equatorial *C*-methyl group (Gorin & Ishikawa, 1967), which is the inverse of that in the formulae in this paper. Pyruvic acid, presumably in the form of ketal, is also a constituent of polysaccharides from *Pseudomonas* species, *Klebsiella rhinoscleromatis* and many *Xanthomonas* species (Gorin & Spencer, 1964).

The other residues shown by methylation analysis in the *E. coli* polysaccharide were 4-linked fucose, 3-linked galactose, 3-linked glucose and 4-linked glucuronic acid. Information about the sequence was derived by partial fragmentation methods. Acid hydrolysis gave 3-*O*-(D-glucopyranosyluronic acid)-D-galactose as a stable product, which is known from other work to have the β-configuration (Rodén & Markovitz, 1966; Sutherland, 1969). When the polysaccharide was converted into the hydroxypropyl ester and then treated with an-



Scheme 2. Base-catalysed cleavage of colanic acid side chains after carboxyl esterification. Note that configurations are assumed for galactosyl bonds and the quaternary carbon atom of the ketal ring. The alkyl group, R, was hydroxypropyl in (II) but was not characterized in the products; it would be methyl or hydroxypropyl, or both, depending on the extent of transesterification.

hydrous base (McCleary *et al.* 1967), fragmentation occurred with the appearance of u.v.-absorption bands that suggested β-elimination at the glucuronate residues. This interpretation was supported by the loss of glucuronic acid residues shown by hydrolysis of a polymeric fraction of the product. The major product of low molecular weight was the monosaccharide derivative, 4,6-*O*-(1′-alkoxycarbonylethylidene)-D-galactose. All the evidence would therefore point to the presence, in the polysaccharide ester, of the feature *O*-(alkoxycarbonylethylidene-D-galactose)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→3)-*O*-D-galactopyranosyl (II) and its degradation by the path shown in Scheme 2. The incomplete release of ketal residues does not necessarily imply that they occur in an additional type of structural situation, for reasons already given (McCleary *et al.* 1967), and which are borne out because the destruction of 4-linked glucuronic acid residues was only partial. The segment (II) occurs as the terminal part of side chains that are joined to fucose branching residues. Various partial structures can be proposed on the basis of this and the remaining methylation evidence, and are discussed more fully in the preceding paper (Sutherland, 1969).

Extracellular polysaccharides that contain galactose, glucose, glucuronic acid and fucose residues are known from other bacteria, and we compared several of this type by methylation analysis. By this criterion, essentially the same polysaccharide was present in *E. coli* K12 S53 slime, *E. coli* K12 S53 capsule, *E. coli* K12 S61 slime, *A. cloacae* slime and *S. typhimurium* slime. It would therefore seem appropriate to use a family name, such as 'colanic acid' (Goebel, 1963), to refer to the whole group. The pattern of methyl glycosides produced by methylation and methanolysis (Fig. 3) can be proposed as a characteristic 'fingerprint'. Physical properties varied widely in the group, both before isolation (e.g. some polysaccharides occurred as soluble slimes but one was a coherent capsule) and afterwards (*E. coli* S61 slime gave very much more viscous solutions than did *E. coli* S53 slime). Some structural variation is therefore likely within the group and 'colanic acid' is to be taken as a generic name rather than as defining a unique substance. Some differences in the natural state might reflect variations in biological organization rather than molecular structure.

Our results suggest that, even between some bacteria that may not be closely related (e.g. *E. coli* and *A. cloacae*), there are close underlying similarities in mechanisms of extracellular polysaccharide biosynthesis, just as, for example, in the synthesis of cell-wall peptidoglycan. Although *S. typhimurium* polysaccharide was unmistakably related to the main group, it differed in giving a slightly lower proportion of ketal glycoside after methanolysis and methylation but, instead, an approximately corresponding amount of 2,3,4,6-tetra-*O*-methylgalactosides. Since the decrease in ketal was measured relative to 2,4,6-tetra-*O*-methylgalactosides and no tri-*O*-methylglucuronic acid derivatives were detected, the tetramethyl ether probably originates from non-reducing termini of the segment (II) that do not carry pyruvic acid. *S. typhimurium* polysaccharide therefore seems to be 'biosynthetically unfinished colanic acid', suggesting that substitution of pyruvic acid occurs after polymerization rather than before.

Another polysaccharide with the same sugar residues as colanic acid, namely the slime from *K. aerogenes* 1.2, was found to be different in the products of methylation and methanolysis. In particular, no peak of high retention time was observed on the gas chromatogram that might have been a fully methylated pyruvic ketal. Such derivatives also appeared to be absent from the cell-wall lipopolysaccharide of *E. coli*. The structure of certain polysaccharides from other *K. aerogenes* strains has since been established in detail (Sandford & Conrad, 1966; Conrad, Bamburg, Epley & Kindt, 1966; Gahan, Sandford & Conrad, 1967).

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