Structural Studies on Colanic Acid, the Common Exopolysaccharide Found in the Enterobacteriaceae, by Partial Acid Hydrolysis

OLIGOSACCHARIDES FROM COLANIC ACID

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The exopolysaccharide slime colanic acid has been isolated from representative strains of *Escherichia coli*, *Salmonella typhimurium* and *Aerobacter cloacae*. Analysis showed that each polymer contained glucose, galactose, fucose and glucuronic acid, together with acetate and pyruvate. The molar proportions of these components were 1:1.8:1.9:1:1 approximately. On the basis of periodate oxidation of the natural and deacetylated polysaccharide, glucose is proposed as the site of the acetyl groups. The pyruvate is attached to galactose. Three neutral oligosaccharides and ten electrophoretically mobile oligosaccharides were isolated and partially characterized. Four of the fragments were esters of pyruvic acid. Most oligosaccharides were isolated from all three polysaccharide preparations. Three further oligosaccharides were isolated from carboxyl-reduced colanic acid and sodium borotritide was used to label the glucose derived from glucuronic acid in these fragments. One trisaccharide was obtained from periodate-oxidized polysaccharide. On the basis of these oligosaccharides a repeating hexasaccharide unit of the following structure is proposed:

The significance of this structure in colanic acid biosynthesis is discussed.

The name colanic acid was applied by Goebel (1963) to an exopolysaccharide slime synthesized by a mucoid *Escherichia coli* strain. This polysaccharide had the same sugar components as material synthesized by several *Salmonella* species (Anderson & Rogers, 1963). In all probability the polysaccharide is the same substance as the M-antigen described by Kauffmann (1954) and frequently reported during studies on the Enterobacteriaceae (e.g. Beiser & Davis, 1957). The control of the production of colanic acid by *E. coli* K12 strains has been the subject of studies by Markovitz and his colleagues (Markovitz, 1964; Markovitz & Rosenbaum, 1965; Kang & Markovitz, 1967). These indicated control by a regulator gene, whose action could be reversed by growth in the presence of *p*-fluorophenylalanine. Thus some non-mucoid strains could be converted to poly-

saccharide production. Studies in this laboratory (Grant, Sutherland & Wilkinson, 1969) have confirmed that a polysaccharide containing glucose, galactose, fucose and glucuronic acid may be synthesized by many species of *Salmonella* and many *E. coli* strains when suitable cultural conditions were employed. Despite the many studies on colanic acid, little knowledge of its structure has been obtained. Analysis of the polysaccharide indicated that the sugar components glucose, glucuronic acid, fucose and galactose were present in the approximate molar proportions 1:1.2:2 (Sapelli & Goebel, 1964). Partial acid hydrolysis of the *E. coli* capsular polysaccharide was also shown to yield two oligosaccharides, one of which was identified as the aldobiuronic acid β -D-glucuronosyl-(1 $\rightarrow$ 3)-D-galactose (Roden & Markovitz, 1966). During studies on colanic acid biosynthesis, it

became necessary to attempt further structural studies on the polysaccharide.

MATERIALS AND METHODS

Bacteria. *Aerobacter cloacae* N.C.T.C. 5920 and *Escherichia coli* S53, a sub-strain of K12, were grown under conditions previously described (Sutherland & Wilkinson, 1965). *Salmonella typhimurium* SL1543 was kindly provided by Dr B. A. D. Stocker, Department of Microbiology, Stanford University, and was grown by the same methods as the other two strains. The polysaccharides of all three strains were excreted as extracellular slime. Capsules were not detected on India ink staining of cultures (Duguid, 1951). The polysaccharides were recovered and purified by using the methods originally employed for the slime polysaccharides of *Klebsiella aerogenes* (Wilkinson, Dudman & Aspinall, 1955). All three products were obtained as white fibrous material, highly hygroscopic and readily soluble in water to give viscous opalescent solutions.

Paper chromatography and paper electrophoresis. All chromatography was performed on Whatman no. 1 paper. The solvent systems were: A, butan-1-ol-acetic acid-water (4:1:5, by vol.) (Partridge, 1946); B, ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.) (Feather & Whistler 1962); C, butan-1-ol-pyridine-water (6:4:3, by vol.) (Whistler & Conrad, 1954); D, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Dörfel, 1955). For paper electrophoresis the buffer used was pyridine-acetic acid-water (5:2:43, by vol.), pH 5.3. Preparative runs were made on Whatman 3MM paper, and other runs on Whatman no. 1 paper. A current of 80–100 mA was applied for 2–4 hr. with 77 cm. × 20 cm. paper strips on a Locarte high-voltage paper-electrophoresis apparatus. For removal of salts or enzyme protein, the same current was applied for 20–30 min.

Microanalytical methods. Glucuronic acid was determined by a modification of the carbazole-H₂SO₄ technique (Bitter & Muir, 1962) on solutions of intact polysaccharide or oligosaccharide. D-Glucose and D-galactose were determined in hydrolysed material by using the respective oxidases. (Glucose oxidase was purchased from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany, and galactose oxidase from Worthington Biochemical Corp., Freehold, N.J., U.S.A.) Fucose was determined by a micro-adaptation of the cysteine-H₂SO₄ procedure. A modification of the method of Hestrin (1949) was used to determine O-acyl residues. The volume of each reagent was reduced to 200 µl. with a resultant final volume of 1 ml. Pyruvate was determined by a modification of the method reported by Sloneker & Orentas (1962). For intact polysaccharide, the material was prepared as a 0.3% solution in M-HCl. For oligosaccharides, 50 µl., containing approx. 5 µg. of pyruvate, was added to an equal volume of distilled water. After the addition of 100 µl. of 2M-HCl the solution was sealed in a hard-glass tube (100 mm. × 5 mm.) and hydrolysed at 100° for 3 hr. Polysaccharide solutions were similarly treated. To each 200 µl. was added 100 µl. of 0.5% 2,4-dinitrophenylhydrazine in 2M-HCl. The mixture was left at room temperature for 5 min. and then 0.5 ml. of ethyl acetate was added. After mixing, the lower aqueous layer was removed with a capillary pipette. The organic phase was extracted with three successive 0.5 ml. portions of aqueous 10% (w/v) Na₂CO₃. The extracts were removed, pooled and made up

to 1.5 ml. before the extinctions were read at 375 nm. in a spectrophotometer. Standards containing 5–10 µg. of pyruvate were included.

All assays were read in glass semi-micro cells in a Zeiss PMQII spectrophotometer.

Borohydride reduction. This was performed on samples containing approx. 0.5 µmole of oligosaccharide in a volume of 50 µl. The NaBH₄ (2.28%) in 0.01 M-KOH was added and the mixture left in stoppered tubes in the dark at room temperature for 12 hr. After excess of borohydride had been destroyed with 2M-acetic acid, salts were removed by electrophoresis. Before analysis, the reduced material was hydrolysed in 0.5M-H₂SO₄ for 4 or 16 hr. at 100° in sealed tubes, the time of hydrolysis depending on whether or not glucuronosyl linkages were present and thus necessitated longer hydrolysis.

Periodate oxidation. This was carried out as described by Conrad, Bamberg, Epley & Kindt (1966). Residual sugars were determined on the material after borohydride reduction and hydrolysis.

Carboxyl reduction. This was carried out by the method of Hungerer, Jann, Jann, Ørskov & Ørskov (1967). After one cycle of reduction, the glucuronic acid content of the polysaccharide from *E. coli* or *A. cloacae* fell from approx. 18% to about 2% with a corresponding rise in the glucose content. The yield was just over 80% of the starting material. Deacylation occurred during the treatment. In one preparation the amount of borohydride was decreased to 1 mg./mg. of polysaccharide and 5 mc of NaBT₄ was included in the mixture to permit differentiation of the glucose formed from that originally present.

Hydrolysis with glycosidases. Three commercial preparations were used: β -glucosidase (EC 3.2.1.21) from L. Light and Co. Ltd., Colnbrook, Bucks.; β -galactosidase (EC 3.2.1.23) and β -glucuronidase (EC 3.2.1.31) from British Drug Houses Ltd., Poole, Dorset. α -Glucosidase (EC 3.2.1.20) was prepared from yeast by the method of Robbins & Uchida (1962). A crude α -fucosidase (EC 3.2.1.-) preparation was made from limpets, *Patella vulgata*, essentially as described by Conchie & Levvy (1957). The (NH₄)₂SO₄-fractionated material possessed strong hydrolytic activity against *p*-nitrophenyl α -L-fucopyranoside as well as other glycosidases. During testing of the activity of all enzymes against oligosaccharide solutions, conditions were used such that approximately equimolar amounts of appropriate substrates such as maltose, cellobiose, lactose or nitrophenyl glucuronide were completely hydrolysed. Where prolonged incubation at 30–35° was required, toluene was added to the incubation mixture to suppress bacterial growth.

RESULTS

Isolation and characterization of the polysaccharides. Each of the polysaccharides was obtained from solid cultures of bacteria in nitrogen-deficient medium. After separation from the cells by high-speed centrifugation, the material was precipitated from the supernatant fluids with cold acetone. The strands of polysaccharide were wound round a glass rod, removed and redissolved in water, dialysed exhaustively and freeze-dried. The colanic acid was redissolved in acetate buffer and thoroughly deproteinized (Sevag, 1934). The material used

Table 1. *Composition of colanic acid*

All values are expressed as % dry wt. —, No valid value obtained.

Bacterial strain	Fucose	Glucose	Galactose	Glucuronic acid	Acetate	Pyruvate
<i>E. coli</i> S53	30.4	18.2	26.0	18.0	6.3	8.6
<i>A. cloacae</i> 5920	28.0	17.9	27.5	17.4	6.0	8.1
<i>S. typhimurium</i> 1543	27.0	17.9	28.8	17.4	5.6	7.9
Modified polysaccharide						
<i>E. coli</i> , oxidized	—	16.7	27.4	0.9	0	5.5
<i>E. coli</i> , alkali-treated	27.3	15.5	27.5	14.5	0	5.1
<i>E. coli</i> , oxidized/alkali-treated	—	4.1	23.0	0.4	0	3.5
<i>E. coli</i> , carboxyl-reduced	28.3	34.0	26.3	2.7	0	2.2

for analysis was dried to constant weight at 100°. Paper chromatography of hydrolysed material confirmed the presence in all preparations of fucose, galactose, glucose and glucuronic acid. Chemical analysis confirmed that the three products were similar with respect to the sugar components (Table 1). As well as the four sugars known to be present, the occurrence of an acyl group was indicated by the hydroxylamine assay. Liberation of the acyl group with alkali followed by characterization of the hydroxamic acid derivative by the technique of Thompson (1951) revealed a spot moving in solvent A with the same R_F value (0.51) as authentic acetylhydroxamic acid. Formylhydroxamic acid moved with R_F 0.43 and was absent from the polysaccharide preparations. Treatment with 0.1M-sodium hydroxide at 60° for 60 min. caused complete loss of the *O*-acetyl groups without affecting the constituent sugars (Table 1). Another component, which has only recently been identified in colanic acid, is pyruvate bound to galactose as a ketal linkage (Lawson *et al.* 1969). Microanalysis for pyruvate indicated that it was present in all the polysaccharide preparations in approximately the same amount (Table 1). Further proof of the similarity of colanic acid derived from the different bacterial strains was seen after treatment of polysaccharide solutions with bacteriophage-induced enzymes (Sutherland & Wilkinson, 1965). The viscosity of each solution was rapidly decreased on incubation at 37°, but in no case were small-molecular-weight products released.

The effects of various chemical treatments on the composition of colanic acid are also shown in Table 1. Although only the results for *E. coli* colanic acid are shown, similar values were obtained for the other preparations.

Periodate oxidation. In all preparations periodate oxidation caused destruction of the glucuronic acid but not of the galactose or glucose. As determined chromatographically, most of the fucose was also unaffected. The results for glucuronic acid destruc-

Fig. 1. Destruction of glucose in alkali-treated colanic acid by periodate.

Fig. 2. Destruction of glucuronic acid by periodate.

tion in colanic acid for *E. coli* are shown in Fig. 1. Some destruction of fucose may have occurred but could not be followed accurately because of the production of material interfering in the cysteine-sulphuric acid reaction. When the polysaccharide was first treated with 0.1M-sodium hydroxide at 56° for 1 hr., then dialysed free of alkali, freeze-dried and oxidized, a slightly different result was obtained. The glucose was rapidly oxidized until after 100 hr. only 10–15% of the original glucose remained. The destruction of glucose in alkali-treated polysaccharide is shown in

Fig. 2. The results of analysis on the oxidized polysaccharides are shown in Table 1.

Partial acid hydrolysis. A 1% (w/v) solution of colanic acid from *E. coli* was added to an equal volume of 0.5M-sulphuric acid and heated at 100°. Samples were removed at 15 min. intervals and neutralized with barium hydroxide. The supernatant fluids were applied to Whatman 3MM paper and subjected to electrophoresis. The most complex pattern of fragments, detected by staining with alkaline silver nitrate, appeared after hydrolysis for 30 min. Longer hydrolysis led to a diminution in the number of charged oligosaccharides, until only one was seen after 120 min. Elution of the neutral material, followed by chromatography in solvent C, showed three neutral fragments moving slower than galactose at 15 min., but only one after 30 min. hydrolysis. From these results, hydrolysis times of 15 and 30 min. were adopted for obtaining neutral and charged oligosaccharides respectively. Each polysaccharide (1g.) was dissolved in 100 ml. of water and added to 100 ml. of acid, then hydrolysed at 100°. Each mixture was divided after 15 min., the first portion being neutralized with Amberlite IR-410 (HCO₃⁻ form) resin and used to obtain neutral oligosaccharides. The second portion was hydrolysed for a further 15 min. and neutralized with barium hydroxide solution. It was then subjected to preparative paper electrophoresis on Whatman 3MM paper. The fractions revealed by staining with alkaline silver nitrate were eluted and checked for purity in one of the acid solvent systems (A or B). Where the material was impure, it was run again in one of these solvents where the separation from contaminating material was best. Although there were slight variations in yield of oligosaccharides from different preparations, the same oligosaccharides were obtained from partial acid hydrolysates of each bacterial polysaccharide unless stated to the contrary. As it was more difficult to obtain sufficient polysaccharide from the *Salmonella typhimurium* strain, only one preparation was made. However, the results with the other two species represent material isolated from several hydrolysates from one polysaccharide preparation of each.

Neutral oligosaccharides. The major neutral

oligosaccharide (N3) obtained from partial acid hydrolysates of colanic acid moved behind galactose in solvent C and was in relatively high yield. Hydrolysis with 0.5M-sulphuric acid followed by neutralization and chromatography in solvent D showed that this fraction contained glucose and fucose in approximately equal amounts. Analysis confirmed that the molar ratio of the two constituent sugars was exactly 1:1. Treatment with sodium borohydride led to complete conversion of fucose into fucitol, indicating that the fraction is a glucosyl-fucose. Treatment with β -glucosidase under conditions where an equimolar amount of cellobiose was completely hydrolysed released 48% of the available glucose. α -Glucosidase had no effect. On electrophoresis in 0.05M-borate buffer, pH 8.5, the fraction moved to the negative pole. When tested with diphenylamine reagent (Bailey & Bourne, 1960), oligosaccharide N3 gave a brownish-green colour differing from the blue colour given by (1 $\rightarrow$ 4)-linked disaccharides such as maltose and cellobiose, and from the brown colour produced by melibiose [α -galactosyl-(1 $\rightarrow$ 6)-glucose]. Chromatography in three solvents and electrophoresis in borate buffer confirmed that oligosaccharide N3 was not identical with an authentic sample of β -glucosyl-(1 $\rightarrow$ 4)-fucose. It must therefore be β -D-glucopyranosyl-(1 $\rightarrow$ 2 or 3)-fucose. Its properties are shown in Table 2.

The second neutral fraction was isolated in much lower yield than oligosaccharide N3. This material (N2) was slower moving than oligosaccharide N3 in chromatographic systems, but hydrolysates contained the same sugars, namely glucose and fucose. The molar ratio of glucose to fucose was 1.2:1, and borohydride reduced 50% of the fucose to fucitol. Treatment with α - or β -glucosidase failed to release any glucose. However, hydrolysis of 0.1 μ mole with 0.125M-sulphuric acid for 10 min. at 100° liberated approx. 54% of the fucose and 3% of the glucose. No unhydrolysed material remained and almost 0.1 μ mole of β -glucosylfucose was recovered by preparative paper chromatography. Thus oligosaccharide N2 is a fucosyl derivative of oligosaccharide N3 and the lack of activity of glucosidase enzymes may indicate the structure as being fucosylglucosylfucose. On treatment with

Table 2. *Neutral oligosaccharides*

Fraction	Yield (μ moles/g. of polysaccharide)	Components	Molar ratio	<i>R</i> _{Glc}			
				Solvent A	Solvent B	Solvent C	Solvent D
N3	19.2	Glc, Fuc	1:1	0.95	0.81	0.74	0.83
N2	1.7	Glc, Fuc	1:2	0.87	0.70	0.26	0.44
N1	3.7	Glc, Fuc	1:3	0.77	0.61	0.21	0.31

limpet α -fucosidase, fucose was released together with β -glucosylfucose. The postulated structure of oligosaccharide N2 is therefore α -L-fucosyl- β -D-glucopyranosylfucose.

The third neutral fragment was present in greater quantity than oligosaccharide N2, and this oligosaccharide (N1) had a lower chromatographic mobility. It also contained glucose and fucose in the molar ratio 1:3. After treatment with sodium borohydride, only 61% of the fucose was recovered along with all of the glucose. Glucose was not released with β - or α -glucosidase. Hydrolysis of 0.2 μ mole, under the same conditions as for oligosaccharide N2, liberated only 5% of the available glucose. The major hydrolysis products were fucose and β -glucosylfucose in the approximate molar ratio 2:1. About 20% of the original oligosaccharide remained unhydrolysed. No trace of material resembling the trisaccharide N2 in its chromatographic mobility could be seen. There was, however, a small amount of material detected on paper chromatography in solvent C with R_{Glc} 1.1. It seems certain that the oligosaccharide N1 is a tetrasaccharide containing three fucose residues and one glucose residue, the glucose being linked to a fucose residue as in oligosaccharide N3. To determine whether the terminal reducing fucose residue was that linked to glucose, 0.2 μ mole was reduced with a mixture of sodium borohydride and sodium borotritide and then hydrolysed with 0.125M-sulphuric acid. On neutralization with resin and paper chromatography in solvent D, the material moving as fucitol and as β -glucosylfucitol, having R_{Glc} 1.90 and 1.11 respectively, was cut from the chromatogram and the associated radioactivity determined in the scintillation spectrometer. All radioactivity, 1161 c.p.m., was associated with the fucitol and none with the reduced disaccharide area. From these results, oligosaccharide N1 is possibly a tetrasaccharide having the structure:

Thus on acid hydrolysis the more labile fucosyl bonds are broken to liberate fucose (2 moles) and β -glucosylfucose (1 mole). The trace of material moving faster than glucose in solvent C may be fucosylfucose.

In addition to the three neutral oligosaccharides described, traces of slower-moving neutral material were obtained in some preparations. Insufficient material was obtained for accurate analysis, but hydrolysates of these fractions always showed the presence of glucose and fucose, there being in every case an excess of fucose over glucose.

Charged oligosaccharides. The pattern of charged oligosaccharides obtained on partial acid hydrolysis was always much more complex than the pattern

of neutral fragments. No preparation contained less than ten electrophoretically mobile oligosaccharides and the yield of some of these was in some cases inadequate to permit satisfactory characterization.

The material obtained in highest yield from all three colanic acid preparations (E3) was found on hydrolysis to contain galactose and glucuronic acid in equimolar amounts. Borohydride treatment converted all the galactose into galactitol and 85% of the galactose was released by β -glucuronidase. The oligosaccharide is therefore considered to be a β -glucuronosylgalactose disaccharide. From its behaviour on paper electrophoresis it could not be distinguished from several aldobiuronic acids with this configuration. However, paper chromatography in solvents B, C and D indicated that it was identical in behaviour with β -D-glucuronosyl-(1 $\rightarrow$ 3)-galactose prepared from heparin and differed from β -D-glucuronosyl-(1 $\rightarrow$ 4)-galactose isolated from *Xanthomonas stewartii* (Gorin & Spencer, 1961a) and β -D-glucuronosyl-(1 $\rightarrow$ 6)-galactose obtained from partial hydrolysis of gum acacia. The oligosaccharide E3 is therefore apparently the same as that isolated earlier from *E. coli* K12 colanic acid by Roden & Markovitz (1966).

Small amounts of a slower-moving oligosaccharide (E5a) also containing galactose and glucuronic acid were obtained. The molar ratio of galactose to glucuronic acid was 2:2:1 by analysis, and pyruvate was absent. Partial acid hydrolysis (0.125M-sulphuric acid at 100° for 20 min.) yielded approximately equal amounts of galactose and β -glucuronosylgalactose. The galactose moiety of the aldobiuronic acid was reduced by borohydride. The enzyme β -glucuronidase had no effect on oligosaccharide E5a, but β -galactosidase released 42% of the available galactose, together with the aldobiuronic acid. It is therefore concluded that fraction E5a is a β -galactosyl-(β -glucuronosylgalactose) trisaccharide.

Two oligosaccharides containing fucose, galactose and glucuronic acid were isolated. On electrophoresis, the faster moving of these, oligosaccharide E4, contained the three sugars in equimolar proportions. On treatment with sodium borohydride, all the fucose was reduced to fucitol. Partial acid hydrolysis (as for oligosaccharide E5a) liberated fucose and the aldobiuronic acid (E3). The enzyme β -galactosidase did not have any effect. Attempts to hydrolyse the fragment with β -glucuronidase led to release of some of the glucuronic acid, but no galactosylfucose was isolated, presumably because of the presence of β -galactosidase detected as a contaminant in the enzyme preparation. The oligosaccharide E4 is thus a trisaccharide of structure β -glucuronosylgalactosylfucose.

The second oligosaccharide containing fucose,

Table 3. *Charged oligosaccharides*

Fraction	Yield (μ moles/g. of polysaccharide)	Components	Molar proportions	M_{GlcUA}	R_{Glc}	
					Solvent C	Solvent D
E1a	2.5	Pyruvate, Gal, GlcUA	1:2:1	0.87	0.28	0.17
E1b	3.0	Pyruvate, Gal	1:1	0.92	1.02	1.95
E2a	1.7	GlcUA, Gal, Glc, Fuc	1:1:1:1	0.69	0.15	0.04
E2b	0.4	Pyruvate, GlcUA, Gal, Fuc	1:1:2:1	0.76	0.32	0.53
E3	38.3	GlcUA, Gal	1:1	0.68	0.43	0.28
E4	0.4	GlcUA, Gal, Fuc	1:1:1	0.60	0.18	0.06
E5a	0.5	Gal, GlcUA	2:1	0.54	0.19	0.08
E5b	1.6	GlcUA, Gal, Fuc	1:1:2	0.55	0.39	0.21
E6	1.2	GlcUA, Gal, Glc, Fuc	1:1:1:2	0.45	0.19	0.07
A1	—	Pyruvate, GlcUA, Gal, Glc, Fuc	1:1:1:1:1	0.64	0.09	0.42

galactose and glucuronic acid (E5b) moved more slowly on electrophoresis but slightly faster in paper-chromatography solvents (Table 3). Analysis indicated fucose, galactose and glucuronic acid in the approximate molar proportions 2:1:1. Of the fucose, 50% was reduced by sodium borohydride. Partial acid hydrolysis yielded fucose, the aldobiuronic acid β -glucuronosylgalactose and the trisaccharide E2b together with trace amounts of galactose and glucuronic acid. The probable structure of oligosaccharide E5b is that of a fucosyl derivative of oligosaccharide E2b, where the structure is either

or

The feature that is difficult to interpret is the relatively fast chromatographic mobility, considering that the fraction appears to be a tetrasaccharide. This may, however, be due to the presence of the two adjacent methylpentose residues. Pyruvate was definitely absent and could not therefore have contributed to the mobility.

A number of charged oligosaccharides were found after hydrolysis to contain fucose, glucose, galactose and glucuronic acid. In one of these (E2a) the component sugars were in the molar proportions 1.0:0.95:0.95:0.8, indicating that it is probably a tetrasaccharide. The terminal reducing sugar as determined by borohydride reduction was fucose. Partial acid hydrolysis (0.125M-sulphuric acid for 20 min. at 100°) released β -glucuronosylgalactose and β -glucosylfucose in equal amounts, with only traces of free sugars. Treatment with β -glucosidase released all the glucose from the oligosaccharide together with a product indistinguishable from the

oligosaccharide E4. The structure of the tetrasaccharide E2a thus seems to be:

This structure seems the most probable as the glucosyl residue can be released enzymically and is also known to be attached to the terminal fucose. Attempts to hydrolyse the complete oligosaccharide with β -glucuronidase were unsuccessful.

A second oligosaccharide with the same constituent sugars (E6) was slower-moving electrophoretically but slightly faster-moving on chromatograms than was oligosaccharide E2a (Table 3). The molar proportions of fucose, galactose, glucose and glucuronic acid were 2:1:1:1 and 47% of the fucose was reduced by sodium borohydride. The products of partial acid hydrolysis were the same as were found for oligosaccharide E2a, namely the aldobiuronic acid and β -glucosylfucose, but there was also a significant amount of fucose released. β -Glucosidase released only 10% of the available glucose. From these results this oligosaccharide appears to be a pentasaccharide analogous to oligosaccharide E5b in structure but containing an additional glucose moiety. Its structure is therefore postulated as:

Two oligosaccharides with very high electrophoretic mobility were isolated in low yield from the *E. coli* and *A. cloacae* preparations, but were not obtained in sufficient amount from the *S. typhimurium* colanic acid to permit accurate analysis. The slower-moving on chromatography (E1a) contained galactose, glucuronic acid and pyruvate

in the molar proportions 2:1:1:1. The terminal reducing sugar was galactose, as borohydride treatment converted 50% of the galactose into galactitol. Both β -galactosidase and β -glucuronidase were inactive towards this oligosaccharide. Partial acid hydrolysates were found on electrophoresis to contain β -glucuronosylgalactose and a fraction equidistant with oligosaccharide E5a. On chromatography in solvent C, a spot moving with the same R_{Glc} as oligosaccharide E1b was observed.

The fraction E1b had very high chromatographic mobility (Table 3), but hydrolysates showed the presence of galactose and pyruvate in the molar ratio 1:1:1. The absence of glucuronic acid was confirmed by the carbazole test, in which a blue colour was formed. All the galactose was converted into galactitol by borohydride. Graded acid hydrolysis failed to yield any product other than galactose. Thus this material is almost certainly pyruvylgalactose. Consequently oligosaccharide E1a has the structure:

It is thus the pyruvyl derivative of oligosaccharide E5a.

A third oligosaccharide containing pyruvate (E2b) was isolated. The sugars detected on chromatography of hydrolysates were fucose, galactose and glucuronic acid in the molar proportions 1:2:1 along with 1 molar proportion of pyruvate. Fucose was the terminal reducing sugar and neither β -galactosidase nor β -glucuronidase caused hydrolysis. Partial acid hydrolysis (0.5M-sulphuric acid for 20 min. at 100°) yielded fucose, galactose, aldobiuronic acid and a trace of pyruvylgalactose.

The largest pyruvylated fragment (A1) was obtained in very low yield from partial acid hydrolysates of colanic acid, but was the major component of autohydrolysates. It contained fucose, glucose, galactose, glucuronic acid and pyruvate and these were present in the molar proportions 1:1:2:1:1. The terminal reducing sugar was fucose and β -glucosidase was the only glycosidase with any effect, releasing 19% of the available glucose. The product had M_{GlcUA} 0.78 on electrophoresis and R_{Glc} 0.55 on chromatography in solvent D. It is therefore similar to the pyruvylated tetrasaccharide (E2b). Partial acid hydrolysis of oligosaccharide A1 yielded pyruvylgalactose, galactose, β -glucosylfucose and the aldobiuronic acid. Its structure is that of a pyruvylated pentasaccharide:

The properties of the charged oligosaccharides are summarized in Table 3.

Oligosaccharides isolated from carboxyl-reduced polysaccharide. The stability of the aldobiuronic acid to acid hydrolysis prevents the isolation of several possible oligosaccharide configurations from the intact polysaccharide. In particular, no neutral galactose-containing fragments were obtained on hydrolysis of colanic acid. As fucose was a branch point, having both glucose and galactose molecules attached to it in several of the oligosaccharides obtained from colanic acid, it seemed important to isolate such oligosaccharides and thereby obtain information on the linear part of the polysaccharide molecule. Such oligosaccharides might be obtained either by converting the glucuronic acid into glucose and thus a more acid-labile glucosyl linkage, or by preferential destruction of the glucuronic acid by periodate oxidation. Both methods were attempted. After carboxyl reduction, colanic acid from *E. coli* or from *A. cloacae* was dissolved in 0.25M-sulphuric acid to give a 1% solution. After hydrolysis for 45 min. at 100°, the material was neutralized with Amberlite IR-410 (HCO_3^- form) resin. Chromatography in solvent B resolved the material into five fractions. The two slowest-moving were each resolved into two components after elution and rechromatography in solvent D. The fractions were named in order of increasing mobility R1a, R1b, R2a, R2b, R3, R4 and R5. Only the oligosaccharides R3 and R4 were obtained in appreciable quantity, and oligosaccharide R5 proved to be identical with pyruvylgalactose (E1b).

To determine the origin of the glucose in the fractions, a partial hydrolysate of tritiated carboxyl-reduced polysaccharide was made by using *E. coli* material. The labelled polysaccharide was prepared as described in the Materials and Methods section and 50 mg. of starting material yielded 43 mg. of product. Of this 35 mg. was hydrolysed under the same conditions as for unlabelled polysaccharide. The same fractions were isolated.

Identity of oligosaccharide R4. Hydrolysates of this oligosaccharide showed the presence of glucose, galactose and fucose and analysis indicated that these sugars were present in equimolar amounts. The terminal reducing sugar determined by borohydride reduction was fucose. Treatment with β -galactosidase had no effect, but β -glucosidase liberated 40% of the available glucose. Chromatographic examination of the hydrolysis products showed fucose, galactose and glucose, presumably owing to the presence of contaminating β -galactosidase activity detected in the emulsin preparation. Acid hydrolysis of 0.1 μ mole (0.125M-sulphuric acid at 100° for 10 min.), followed by neutralization and chromatography in solvent A, revealed fucose, glucose, galactose and a spot moving slightly more

Table 4. *Oligosaccharides from carboxyl-reduced colanic acid*

(A) Unlabelled fractions

Fraction	Yield (μ moles/g. of polysaccharide)	Component sugars	Molar proportions	R_{Glc}			
				Solvent A	Solvent B	Solvent C	Solvent D
R4	9.9	Glc, Gal, Fuc	1:1:1	0.80	0.60	0.25	0.56
R3	14.0	Glc, Gal, Fuc	2:1:1	0.59	0.26	0.05	0.24
R2a	0.5	Glc, Gal, Fuc	2:2:1	0.34	0.14	0.01	0.08

(B) Tritiated fractions

Fraction	Sp. radioactivity (c.p.m./ μ mole)	Glucose content (μ moles/ μ mole of oligosaccharide)	
		Radioactivity	Glucose oxidase
Glucose	39	1.0	1.0
R4	89	2.3	1.0
R3	31	0.8	2.0
R2a	43	1.1	2.0

slowly than β -glucosylfucose. Elution and hydrolysis of this material from a duplicate chromatogram showed it to be composed of galactose and fucose. To determine the source of the glucose in oligosaccharide R4 the corresponding tritiated fraction was examined. The specific radioactivity of the glucose (c.p.m./ μ mole) from the tritiated carboxyl-reduced polysaccharide is shown in Table 4, as are those of the oligosaccharides. If the glucose is derived from that originally present, no radioactivity should be present. The specific radioactivity of the glucose from the polysaccharide will be 50% of that derived from the glucuronic acid, as glucose and glucuronic acid were originally present in equimolar amounts. Table 4 shows that the glucose in oligosaccharide R4 has more than twice the specific radioactivity of the glucose isolated from the polysaccharide. It must therefore be derived entirely from glucuronic acid. The value of 2.3 is presumably accounted for by the incomplete conversion of glucuronic acid into glucose. Thus oligosaccharide R4 corresponds to the oligosaccharide E4, glucuronosylgalactosylfucose, and its structure is β -glucosyl-(1 $\rightarrow$ 3)-galactosylfucose.

The oligosaccharide obtained in highest yield from the carboxyl-reduced polysaccharide (R3) also contained fucose, glucose and galactose. The fucose was in the terminal reducing position. Analysis showed that the sugars were present in the molar proportions 1:2:1. Acid hydrolysis of 0.1 μ mole under the same conditions as for oligosaccharide R4 liberated material indistinguishable from oligosaccharide R4, together with β -glucosylfucose, galactose, glucose and fucose. A small amount of material with R_{Glc} 0.35 in solvent B was also detected. Elution of this followed by hydrolysis and chromatography in solvent A indicated approximately equal amounts of glucose and

galactose. Treatment of oligosaccharide R3 with β -glucosidase released about 20% of the available glucose. Chromatography showed the formation of two new fragments. One was indistinguishable from oligosaccharide R4. The other had R_{Glc} 0.43 in solvent B and contained equimolar amounts of glucose, galactose and fucose. The tritiated material had almost the same specific radioactivity as had glucose (Table 4) and so was derived equally from glucose and glucuronic acid in the polysaccharide. These results indicate that oligosaccharide R3 is a tetrasaccharide of structure:

The oligosaccharide R2a contained glucose, galactose and fucose in the molar proportions 2:2:1, and partial acid hydrolysis yielded β -glucosylfucose and oligosaccharides R3 and R4 as the major products. Insufficient material was available to permit further characterization. The tritiated fraction showed specific radioactivity similar to that in oligosaccharide R3, indicating equal derivation of the glucose from glucose and glucuronic acid. The most likely structure is that of the β -galactosyl derivative of oligosaccharide R3. The other fragments were in such low yield that characterization was not possible. The main properties of the R series of oligosaccharides are summarized in Table 4.

Oligosaccharides from oxidized polysaccharide. Three neutral oligosaccharides were isolated from oxidized colanic acid after reduction and hydrolysis with 0.5M-sulphuric acid for 15 min. at 100°. The fragments were separated by paper chromatography in solvent B. The fastest-moving oligosaccharide was identical in all respects tested with β -glucosylfucose obtained from other preparations. A trace

of material moving slightly more slowly proved on hydrolysis to contain equal amounts of galactose and fucose.

The third fragment had R_{Glc} 0.42, 0.19 and 0.24 in solvents B, C and D respectively. It contained equimolar amounts of glucose, galactose and fucose, the fucose being the terminal reducing sugar. Partial acid hydrolysis, as for the R fractions, released galactose and β -glucosylfucose. The enzyme β -galactosidase released 48% of the available galactose along with β -glucosylfucose, whereas only 10% of the glucose was removed by β -glucosidase. It seems probable that this oligosaccharide has the structure:

DISCUSSION

Although many bacterial strains synthesize polysaccharide capsules and slime, the structure of only a few of these polymers is known. In the genus *Klebsiella*, several of the polysaccharides have proved to be based on a tetrasaccharide repeating unit in which one of the sugars forms a short side chain to the main linear molecule. Such structures are seen in *Klebsiella* type 2 (Gahan, Sandford & Conrad, 1967) and type 54 (Conrad *et al.* 1966). In the latter substance, the structure proved rather more complex in that every other tetrasaccharide was acetylated and a further non-carbohydrate substituent, as yet unidentified, was attached to each tetrasaccharide (Sutherland, 1967; Sutherland & Wilkinson, 1968). A knowledge of the structure of colanic acid, widely secreted by several genera of the Enterobacteriaceae, together with the accumulated knowledge on genetic control in this family, should provide a useful system for the study of polysaccharide biosynthesis.

The material named colanic acid by Goebel (1963) contained glucose, galactose, fucose and glucuronic acid. Many workers have since reported the production of a polysaccharide of this chemotype by species of the Enterobacteriaceae but have provided no evidence that the material from different species is identical in composition and structure. Indeed the only portion of the colanic acid structure so far elucidated was the β -glucuronosyl-(1 $\rightarrow$ 3)-galactose identified in *E. coli* K12 material (Roden & Markovitz, 1966). Colanic acid from different species and strains was affected by bacteriophage-induced enzymes, the viscosity of the polysaccharide solutions being greatly decreased (Sutherland & Wilkinson, 1965). However, the lack of small-molecular-weight products precluded any conclusions about the identity or otherwise of the polysaccharide from *E. coli* and *A. cloacae*.

No mention was made in earlier work of acyl groups, although a brief report mentions their presence in a polysaccharide from an *E. coli* strain obtained from pathogenic material and probably resembling colanic acid (Linker & Evans, 1968). The present study confirms that *O*-acetyl groups are present in colanic acid in the molar ratio 1:1 with glucose or glucuronic acid. The lability of the acyl linkage and the absence of enzymes causing complete hydrolysis of the polysaccharide prevented isolation of *O*-acetylated oligosaccharides such as these derived from *K. aerogenes* type 54 and *E. coli* K27 polysaccharides (Sutherland & Wilkinson, 1968; I. W. Sutherland, K. Jann & B. Jann, unpublished work). This made identification of the site of acetylation difficult. However, the destruction of glucose with periodate in alkali-treated polysaccharide is indicative that glucose is the site of the *O*-acetyl group. On the other hand, chemical evidence (Lawson *et al.* 1969) indicates that the glucose is bound at the 3-position and should therefore be periodate-resistant, even after removal of the acyl group. This point thus remains unclear.

Before its identification by n.m.r. spectroscopy (Lawson *et al.* 1969) pyruvylated galactose was not known to be part of the colanic acid polymer. Although pyruvylgalactose was reported as a constituent of agar (Hirase, 1957) it has only once been reported previously in a bacterial polysaccharide (Gorin & Spencer, 1961b). The first report of pyruvate in a ketal linkage in bacterial material was made by Sloneker & Orentas (1962) for *Xanthomonas campestris*. The pyruvylated sugar moiety in this polysaccharide proved to be glucose. A further examination indicated that several polysaccharides synthesized by *Xanthomonas* species were both pyruvylated and acetylated (Orentas, Sloneker & Jeanes, 1963). In the present study, the relative stability of the ketal linkage has permitted isolation of the sugar to which it is attached and also several pyruvate-containing fragments. The pyruvylgalactose isolated from colanic acid was obtained after mild acid hydrolysis, whereas the conditions necessary for breaking the glucuronosyl linkages are much harsher. A pyruvylated aldobiuronic acid was not isolated. It is therefore concluded that the pyruvylated galactose molecule is not the same galactose residue that forms the reducing terminal of the aldobiuronic acid β -glucuronosylgalactose.

From the results of analyses it is difficult to obtain good molar proportions for the polysaccharide components and hence to deduce a possible repeating unit. It seems probable that glucose, glucuronic acid, acetate and pyruvate are present in equimolar amounts. Similarly the ratio of galactose to fucose is approximately 1:1. The determination of galactose by the galactose oxidase method may well have given low values as a result of the incomplete

hydrolysis of the aldobiuronic acid. If both galactose and fucose values are low, a possible repeating unit would be a hexasaccharide containing one residue each of glucose, glucuronic acid, acetate and pyruvate and two residues each of fucose and galactose. A hexasaccharide was not isolated, but a pentasaccharide (E6) was obtained. The structure postulated for this oligosaccharide, together with the fact that pyruvylgalactose is known to be distal to the aldobiuronic acid, leads to the postulation of a repeating unit with the configuration:

Almost all the possible fragments from this proposed repeating unit have been characterized among the oligosaccharides obtained by partial acid hydrolysis. In addition, the tetrasaccharide α -fucosylglucosylfucosylfucose (N1) may give an indication of the structure of the linear chains of the colanic acid molecule. The terminal non-reducing fucose of this tetrasaccharide was presumably derived from the adjacent hexasaccharide unit. The positions of the linkages to glucuronic acid, glucose and fucose are shown by methylation analysis (Lawson *et al.* 1969). The presence in low yield of other, larger, fragments containing only glucose and fucose is indicative of a main chain composed of these sugars, presumably in the ratio of one glucose residue to two fucose residues throughout. It would thus be of the form:

The lability of the fucosylfucose linkage to acid probably accounts for the failure to isolate a disaccharide composed of fucose. The trisaccharide side chains are attached to the fucose residues bound β -glucosidically. Charged oligosaccharides containing more than one aldobiuronic acid or pyruvylgalactose were not detected. If this observation is correct, the side chains attached to the alternate fucose molecules are limited to trisaccharides each containing two galactose residues, one glucuronic acid residue and one pyruvate residue.

The repeating unit proposed for colanic acid is

somewhat more complex than those identified so far in other bacterial extracellular polysaccharides. This implies a requirement for a considerable amount of genetic information. Of the sugars comprising colanic acid, two, fucose and glucuronic acid, are not known to be present in other polysaccharides synthesized by the parent bacteria. On the other hand, glucose and galactose are involved in the lipopolysaccharides of *E. coli* and *S. typhimurium*, although apparently in *A. cloacae* N.C.T.C. 5920 only glucose is present (Sutherland & Wilkinson, 1966). Glucose is also involved in glycogen, although the utilization of ADP-glucose as the activated donor for this system effectively differentiates it from UDP-glucose-requiring systems such as those synthesizing lipopolysaccharide and presumably exopolysaccharide. Acetate and pyruvate are obviously involved in many other cell reactions, but a requirement for specific transferase enzymes must be postulated. The activated donor for acetate is presumably acetyl-CoA and that for pyruvate may be phosphoenolpyruvate. Apart from the enzymes required for formation of the activated donors such as UDP-glucose, UDP-galactose, UDP-glucuronic acid and GDP-fucose, the postulated repeating unit would require eight transferases. The role of the acetate and pyruvyl groups remains obscure. They could perhaps be involved in the control of colanic acid synthesis. Thus addition of pyruvate might terminate synthesis of the side chain at the lipid intermediate stage known to be involved in polysaccharide synthesis (Troy & Heath, 1968). In the same way acetylation might signal the transfer of a complete hexasaccharide unit from a lipid intermediate to another acceptor. Although many bacterial exopolysaccharides do not contain acetyl or pyruvyl groups, most of those so far identified have a simpler structure than colanic acid.

The genetic control of colanic acid biosynthesis in *E. coli* has been the subject of several studies by Markovitz and his colleagues (Markovitz, 1964; Markovitz & Rosebaum, 1965; Kang & Markovitz, 1967). These indicated that a regulator gene controlled the synthesis of colanic acid and of several enzymes thought to be involved in the process. Mucoid production could be induced by de-repressing the regulator with fluorophenylalanine. This feature is known to be widespread in *E. coli* and *S. typhimurium* strains and among other *Salmonella* species (Grant, 1968). An episomal element was postulated by Markovitz & Rosenbaum (1965) as containing the regulator gene and this might account for the widespread occurrence of colanic acid within the Enterobacteriaceae.

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