The Exopolysaccharide of *Klebsiella aerogenes* A3 (Sl) (Type 54) THE ISOLATION OF O-ACETYLATED OCTASACCHARIDE, TETRASACCHARIDE AND TRISACCHARIDE

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The exopolysaccharide slime produced by *Klebsiella aerogenes* A3 (Sl) (type 54) is an O-acetylated polysaccharide, the components of which are glucose, glucuronic acid, fucose and acetyl in the molar proportions 4:2:2:1. A phage-induced fucosidase was obtained that hydrolyses the polysaccharide to give an octasaccharide having the same constituents in the same molar proportions. This octasaccharide (O3) is considered to be the repeating unit of the polysaccharide. It is hydrolysed by other phage-induced fucosidases described earlier to release two tetrasaccharides (O1 and O2). These differ only in that tetrasaccharide O2 is acetylated. An acetylated trisaccharide of structure β -glucosylglucuronosylfucose was prepared from tetrasaccharide O2. A further unidentified group is present. Cell-free preparations were used to acetylate the disaccharide α -glucuronosylfucose. From these results the structure of the octasaccharide (O3) is postulated and its significance in the biosynthesis of the polysaccharide discussed.

The presence of O-acetyl groups in bacterial polysaccharides has been known for a considerable time, the exopolysaccharide capsule of *Diplococcus pneumoniae* type I being one of the first examples reported (Avery & Goebel, 1933). Since then, many O-acetylated polysaccharides have been found, and pyruvate linked as a ketal has also been detected (Sloneker & Jeanes, 1962; Govin, Ishikawa, Spencer & Sloneker, 1967). A problem in all the studies on O-acetylation is the extreme lability of the acetyl groups to acid or alkali. Identification has frequently depended, as in several of the pneumococcal polysaccharides, on immunochemical methods (e.g. Estrada-Parra & Heidelberger, 1963). Most bacterial polysaccharides have been examined by the classical chemical techniques involving methylation, periodate oxidation and acid hydrolysis, during which any acetyl or other O-substituents originally present are hydrolysed and released. Further, the paucity of enzymes catalysing the hydrolysis of bacterial polysaccharides may explain the apparent absence of isolation methods for O-acetylated oligosaccharides.

A good example of the limitations of the older chemical techniques is seen with the polysaccharide of *Klebsiella aerogenes* A3 (Sl) (type 54). No mention of acetyl groups was made in the initial chemical studies on this material (Wilkinson, Dudman & Aspinall, 1955). However, the presence of 4.0% acetate was indicated by the immunochemical studies of Davies, Crumpton, Macpherson & Hutchinson (1958). Recent chemical studies (Sandford & Conrad, 1966; Conrad, Bamberg,

Scheme 1. Suggested octasaccharide repeating unit of *K. aerogenes* A3 polysaccharide. Abbreviations: GlcUA, glucuronic acid; Fuc, fucose; Ac, acetyl; X, unknown group.

Epley & Kindt, 1966) again make no mention of any acetyl groupings.

In an earlier paper (Sutherland, 1967) phage-induced fucosidases were used to hydrolyse the polysaccharide and the presence of an acetyl group on one of the oligosaccharides so obtained was postulated on the basis of chromatographic mobilities and other data. It was suggested that the structure of the polysaccharide was based on an octasaccharide repeating unit (Scheme 1). The site of attachment of the acetyl group was unknown and the occurrence of a further group (X) of unknown characteristics, at a frequency of one per four monosaccharide units, was also indicated. The present paper describes further studies on the acetyl group in the *K. aerogenes* A3 slime polysaccharide and the isolation of three O-acetylated fragments by enzymic hydrolysis.

MATERIALS AND METHODS

Bacteria and bacteriophages. *K. aerogenes* A3 (Sl) and its method of culture for enzyme production and for polysaccharide production were described by Sutherland (1967). The isolation and culture of phage F34 were also described

by Sutherland (1967). A further enzyme-inducing phage F 39 was isolated from sewage by the methods described by Sutherland & Wilkinson (1965) for isolating depolymerase-inducing bacteriophages active against *Escherichia coli*. Enzyme was also prepared from phage F 39-infected culture of *K. aerogenes* A3 (SI) by these methods and partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Analytical methods and borohydride reduction. The techniques were those described by Sutherland (1967).

Paper electrophoresis. The buffer used contained pyridine-acetic acid-water (5:2:43, by vol.), pH 5.3. Strips of Whatman no. 1 paper (77 cm. x 20 cm.) were used for all runs. The separation of charged oligosaccharides in comparative runs was satisfactory after application of 80–100 ma for 4–5 hr. For removal of salts or enzyme proteins, the same current was applied for 30 min. All electrophoresis was performed on a Locarte high-voltage paper-electrophoresis equipment.

Paper chromatography. Descending paper chromatography on Whatman no. 1 paper was used with the following systems: *A*, butan-1-ol-pyridine-water (6:4:3, by vol.) (Whistler & Conrad, 1954); *B*, ethyl acetate-acetic acid-pyridine-water (5:1:5:3, by vol.) (Fischer & Dörfel, 1955); *C*, ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.) (Feather & Whistler, 1962); *D*, butan-1-ol-acetic acid-water (4:1:5, by vol.) (Partridge, 1946). The irrigation times were normally 48 hr., 20 hr., 24 hr. and 120 hr. respectively. Sugars and oligosaccharides were detected with alkaline AgNO_3 reagent (Trevelyan, Procter & Harrison, 1950).

Carboxyl reduction. The method of Hungerer, Jann, Jann, Ørskov & Ørskov (1967) was used. After reduction of the bacterial polysaccharide the yield was 83% of the starting material. Analysis of a typical preparation showed a decrease in the uronic acid content from about 20% to about 3%.

O-Acetyl determination. A micro-adaptation of the method of Hestrin (1949) was used with the volumes of each reagent decreased to 200 μl . and a resulting final volume of 1 ml. The colour obtained was measured at 540 m μ against a reagent blank in the micro-cells of a Zeiss PMQ II spectrophotometer. A solution (0.04 M) of acetylcholine was used as standard.

Preparation of ^{14}C acetyl-CoA. CoA (C. F. Boehringer

und Soehne G.m.b.H., Mannheim, Germany) was acetylated with $[1-^{14}\text{C}]$ acetic anhydride obtained from The Radiochemical Centre, Amersham, Bucks. The method was that described by Ochoa (1957). The product was diluted with acetyl-CoA (Sigma Chemical Co., St Louis, Mo., U.S.A.) to give a final specific radioactivity of approx. 20 mc/m-mole.

Radioactivity assay. Counts were made in a Beckman liquid-scintillation spectrometer with a dioxan-based scintillation fluid (NE572; Nuclear Enterprises Ltd., Edinburgh).

RESULTS

Evidence for the acetylation of K. aerogenes A3 exopolysaccharide. The first indication of the presence of O-acetyl groups in the slime polysaccharide of *K. aerogenes* A3 was the observation by Davies *et al.* (1958) that treatment with dilute alkali led to a 4% loss in weight of the polysaccharide. This was accompanied by changes in immunological properties. Further indication of the possible presence of an O-acetyl group was the isolation of a tetrasaccharide with relatively high chromatographic mobility (Sutherland, 1967) after digestion of the polysaccharide with phage-induced fucosidases.

Analysis of the intact polysaccharide by the ferric chloride-hydroxylamine technique (Hestrin, 1949) indicated the presence of an acyl grouping. When calculated as acetyl, the molar proportions of the components of the polysaccharide were glucose:glucuronic acid:fucose:acetyl, 4:2:2:1. These proportions are in good agreement with the structure postulated by Sutherland (1967) and with the results of Davies *et al.* (1958). Comparison of the absorption curves with the ferric chloride-hydroxylamine reaction showed that the polysaccharide and acetylhydroxamic acid both gave λ_{max} at 505 m μ . The acyl group was identified as acetyl by the method of Thompson (1951). The acetylhydroxamic acid was prepared from the

Table 1. *Properties of oligosaccharides obtained by enzymic hydrolysis of K. aerogenes A3 (SI) (type 54) polysaccharide*

Oligosaccharides O1, O2 and O3 were obtained by the action of phage-induced fucosidases. Oligosaccharides O1c and O2c were obtained by the action of 'cellulase' on oligosaccharides O1 and O2, and oligosaccharide O3a was obtained by treatment of oligosaccharide O3 with 0.01 N-KOH. Details are given in the text. The terminal reducing sugar of all these fractions was fucose. Abbreviations: GlcUA, glucuronic acid; Fuc, fucose; Ac, Acetyl; M_{GlcUA} electrophoretic mobility relative to glucuronic acid.

Oligosaccharide	Component	Molar proportions	M_{GlcUA}	R_{Glc} in solvent			
				<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
O1	Glc, GlcUA, Fuc	2:1:1	0.48	0.05	0.49	0.08	0.01
O2	Glc, GlcUA, Fuc, Ac	2:1:1:1	0.45	0.07	0.63	0.18	0.03
O1c	Glc, GlcUA, Fuc	1:1:1		0.46	0.55	0.20	0.28
O2c	Glc, GlcUA, Fuc, Ac	1:1:1:1		0.45	0.68	0.20	0.29
O3	Glc, GlcUA, Fuc, Ac	4:2:2:1	0.49	0.02	0.03	0.01	0.01
O3a	Glc, GlcUA, Fuc	4:2:2	0.52	—	0.02	—	—

polysaccharide and run in solvent *D* for 20 hr. The paper was sprayed with 10% (w/v) ferric chloride solution at room temperature. An intense purple spot appeared with R_F 0.51, identical with that of the spot of the authentic derivative prepared from ethyl acetate. A very faint spot with R_F 0.43 was also observed, moving with formylhydroxamic acid. It is not known whether a small proportion of formyl residues was present on the polysaccharide or whether this was an artifact. The latter explanation is more probable, as similar results have been reported with the *O*-acetylated extracellular polysaccharide of *Xanthomonas campestris* (Sloneker & Jeanes, 1962).

Isolation of the octasaccharide repeating unit. The phage-induced enzymes used previously (Sutherland, 1967) gave two products after hydrolysis of *K. aerogenes* A3 (Sl) exopolysaccharide. These were the two tetrasaccharides, O1 and O2, whose properties are shown in Table 1. Another phage, F39, has recently been isolated in our Laboratory and is also capable of inducing a polysaccharide depolymerase in the host cells. Preparations of the enzyme induced by phage F39 were found to hydrolyse the polysaccharide solutions and release diffusible material. Chromatographic examination in several solvents showed the production of trace amounts of the tetrasaccharides O1 and O2, together with a large amount of slower-moving material. Preparative chromatograms were run in solvent *B* and the slow-moving material was eluted as a chromatographically pure preparation. The chromatographic and electrophoretic properties of this fragment, designated oligosaccharide O3, are shown in Table 1. Microanalysis showed the presence of glucose, glucuronic acid, fucose and acetyl in the molar proportions 4:2:2:1. Borohydride reduction led to the conversion of half the fucose content into fucitol. It thus seemed probable that oligosaccharide O3 was an octasaccharide with fucose as the terminal reducing sugar and one acetyl residue/mol. Oligosaccharide O3 was resistant to hydrolysis with β -glucosidase or β -glucuronidase. Treatment with 0.01N-potassium hydroxide for 16 hr. at 20° resulted in loss of the acetyl group. The deacetylated material (O3a) had electrophoretic and chromatographic mobilities slightly different from those of oligosaccharide O3 (Table 1).

The structure of oligosaccharide O3 was revealed by the action of the fucosidase induced by phage F34. This enzyme was allowed to act at pH 7.5 and 30° for 16 hr. The enzyme protein and salts were removed by electrophoresis. Chromatography of the enzyme hydrolysate in solvent *B* then showed the presence of two fractions, which were identical, in every property tested for, with tetrasaccharides O1 and O2 respectively. The two oligosaccharides were produced in equal amounts. The resistance of

oligosaccharide O3 to hydrolysis by enzymes other than the phage-induced fucosidases rendered further identification difficult.

It was not known whether the fucose residue reduced by borohydride was part of an O1 or O2 portion of the octasaccharide. Attempts to subject the polysaccharide to carboxyl reduction followed by cellulase treatment were unsuccessful, as the polysaccharide was deacetylated during the process. Similarly borohydride reduction by the standard technique led to production of a reduced form of material O3a. Reduction without deacetylation was achieved by dissolving the material in 1M-N-potassium hydroxide instead of the 0.01N-potassium hydroxide used in the normal procedure. The reduced oligosaccharide O3 was freed from salts by electrophoresis and treated with phage F34-induced enzyme at 30° for 16 hr. The products were freed from protein and salts by electrophoresis followed by chromatography in solvent *B*. Two oligosaccharides were detected, the faster-moving being acetylated and identical with tetrasaccharide O2 in all respects tested. The other fraction contained fucitol and stained weakly with alkaline silver nitrate. Its chromatographic mobility was the same as that of reduced tetrasaccharide O1. It thus seems likely that oligosaccharide O3 is composed of equal amounts of the two tetrasaccharides O1 and O2, where the terminal non-reducing sugar is part of the latter moiety.

Acetylated tetrasaccharide. Analysis of tetrasaccharides O1 and O2 had earlier shown that both were essentially tetrasaccharides of the general structure β -cellobiosylglucuronosylfucose. However, neither was identical with the compound of this structure isolated after mild acid hydrolysis of the polysaccharide. One reason for this could be the presence of acetyl groups, which would be lost on acid hydrolysis. Analysis showed the presence of acetyl in one fraction only, O2, there being one acetyl group/mol. of oligosaccharide (Table 1). This acetyl group could be removed with 0.01N-alkali at room temperature, yielding material indistinguishable from tetrasaccharide O1 (Table 1).

Acetylated trisaccharide. Previous attempts to form trisaccharides from tetrasaccharides O1 and O2 by using 'cellulase' preparations were unsuccessful. However, by using a fresh batch of 'cellulase' (British Drug Houses Ltd., Poole, Dorset) and digestion at pH 7.0 (0.02M-phosphate buffer) for 16 hr., glucose was released from the tetrasaccharide O2. Analysis showed that 50% of the available glucose was released. A similar result was obtained with the non-acetylated fraction O1. In addition to glucose, each tetrasaccharide released a larger fragment, these being designated O2c and O1c respectively. Each of these contained glucose, glucuronic acid and fucose in the molar proportions

Scheme 2. Production of oligosaccharides from *K. aerogenes* A3 slime. Abbreviations: GlcUA, glucuronic acid; Fuc, fucose; Ac, acetyl; X, unknown group. The value of 19–20 repeating units is calculated from data of Conrad *et al.* (1966).

1:1:1 (Table 1). The oligosaccharide O2c also contained an acetyl group. In both cases, the terminal reducing sugar was fucose (Table 1). As was shown with the corresponding tetrasaccharides, treatment with dilute alkali converted oligosaccharide O2c into oligosaccharide O1c. The relationships between these different fractions and the ways in which they can be interconverted are shown in Scheme 2. Neither of the two trisaccharides was identical with the trisaccharide β -glucosylglucuronosylfucose obtained by mild hydrolysis of the polysaccharide (Sutherland, 1967). This confirms the belief (Sutherland, 1967) that some other residue, as yet unidentified, is present in both oligosaccharides O1c and O2c.

Attempts to break down the trisaccharides with enzymes were unsuccessful. Although it might be expected that β -glucosidase would release glucose and disaccharide from oligosaccharide O2c or O1c, no hydrolysis occurred. This has been ascribed by Conrad *et al.* (1966), in experiments with the corresponding trisaccharide β -glucosylglucuronosylfucose, to the aglycone specificity of the enzyme preparation. The unidentified group (X in Scheme 2) may also cause steric hindrance, thus further inhibiting enzyme action.

Site of acetylation. From the analytical data, it was apparent that the *O*-acetyl group was present in tetrasaccharide O2, but absent from tetrasaccharide O1. The terminal glucosyl residue is obviously not acetylated, as 'cellulase' treatment yielded glucose and an acetylated trisaccharide. The point of attachment of the acetyl group is therefore limited to two positions on each of the three sugar residues, all other positions being involved in glycosidic linkages in the intact polysaccharide (Conrad *et al.*

1966). The resistance of the trisaccharide to enzymic hydrolysis prevented isolation of an acetylated monosaccharide by this means. Attempts to produce a susceptible substrate by carboxyl reduction of tetrasaccharide O2 released the acetyl group. An alternative approach was the use of [^{14}C]acetyl-CoA, assumed to be the precursor of the acetyl group. As enzyme, an ultrasonic lysate of exponential-phase cells of *K. aerogenes* A3 (Sl) grown in 0.5% (w/v) glucose broth was used. Unbroken cells were removed by centrifugation at 10000g for 15 min. The supernatant fluid was ultracentrifuged at 25000g for 30 min. The gelatinous sediment was used without further purification. It was suspended in 0.02M-disodium hydrogen phosphate-potassium dihydrogen phosphate buffer, pH 7.2, at a concentration of approx. 5mg. of protein/ml. The 'enzyme' was added to a mixture of [^{14}C]acetyl-CoA and substrate in a total volume of 50 μl . The mixture was incubated at 37° for 30 min., then applied to strips of Whatman no. 1 paper. These were subjected to electrophoresis then cut longitudinally in 1 cm. strips. Each was scanned in a Tracerlab 4Pi scanner. The radio-active peaks were cut out, eluted and counted in the Beckman liquid-scintillation spectrometer. The results are shown in Table 2. The substrates used were obtained by enzymic hydrolysis (oligosaccharides O1 and O1c) or by autohydrolysis as described by Sutherland (1967). The α -glucosylfucose was prepared from carboxyl-reduced polysaccharide by hydrolysis with N-sulphuric acid for 15 min. It was isolated from the hydrolysate by electrophoresis and chromatography of the neutral material in solvent A. It had R_{Glc} 0.95 in that solvent, moving with galactose. It is apparent that

Table 2. *Enzymic acetylation of oligosaccharides*

The 'enzyme' was a particulate fraction produced by ultrasonic lysis of *K. aerogenes* A3 (Sl). Details are given in the text. Mixtures of crude 'enzyme' and substrate oligosaccharides were incubated at 37° for 30 min.

Substrate (200 μ moles)	% of available ^{14}C incorporated from [^{14}C]acetyl-CoA
α -Glucosylfucose	1.4
α -Glucuronosylfucose	13.8
β -Glucosylglucuronosylfucose	3.2
β -Cellobiosylglucuronosylfucose	1.1
Oligosaccharide O1 (tetrasaccharide)	1.0
Oligosaccharide O1c (trisaccharide)	1.0
Fucose	< 1.0
Glucuronic acid	< 1.0

much more radioactivity was transferred to the disaccharide α -glucuronosylfucose than to any of the other substrates tested. This would seem to indicate that either the fucose or glucuronic acid is acetylated.

An attempt to characterize the acetylation product was made by using ten times as much enzyme and α -glucuronosylfucose and replacing the labelled material by 0.25 m-mole of acetyl-CoA (Sigma Chemical Co.). After incubation the mixture was divided into two equal portions and subjected to the standard electrophoretic separation. One strip was stained with alkaline silver nitrate and revealed two spots. The fastest moved with authentic α -glucuronosylfucose. The slower-moving had M_{GlcUA} 0.37. The area in the duplicate strip was eluted and applied to a chromatogram run in solvent C. It had R_{Glc} 0.83 compared with R_{Glc} 0.67 for the unacetylated disaccharide and was chromatographically homogeneous.

DISCUSSION

The presence and role of *O*-acetyl as distinct from *N*-acetyl groups in bacterial polysaccharides appears to have received relatively little study. Only by using an immunochemical approach has their importance been shown, both in exopolysaccharides such as those of *D. pneumoniae* (Heidelberger, Estrada-Parra & Brown, 1964) and in lipopolysaccharides such as those of *Salmonella* group B (Kotelko, Staub & Tinelli, 1961). Investigations of the changes involved in the chemistry of *Salmonella anatum* lipopolysaccharide effected by lysogenic conversion (Robbins & Uchida, 1965; Robbins, Keller, Wright & Bernstein, 1965) also revealed the importance of hitherto unsuspected *O*-acetyl groups.

Although the acyl groupings most frequently

found in bacterial polysaccharides are acetyl residues, other groups such as pyruvoyl have been recorded. Indeed the polysaccharide of *X. campestris* appears to be unusual in that it contains both pyruvate and acetate (Sloneker & Jeanes, 1962). However, the presence of pyruvoyl residues in the ketal linkage as found in *X. campestris* polysaccharides can be excluded from the present consideration, as acid hydrolysates of *K. aerogenes* A3 polysaccharide showed no detectable pyruvate.

The identification of the site of acetylation is attended by several problems. The most difficult to overcome is the extreme lability of *O*-acetyl groups to acid and alkali. The situation is not eased by the lack of enzymes degrading bacterial polysaccharides such as that excreted by *K. aerogenes* A3 (Sl). The isolation of several bacteriophages capable of inducing specific fucosidases has been of great assistance. The difference between the two types of fucosidases hydrolysing the polysaccharide into a tetrasaccharide or octasaccharide unit proves the occurrence of acetyl groups on alternate tetrasaccharide units. Both hydrolases will attack the deacetylated polysaccharide, producing tetrasaccharide units. Therefore the difference in specificity of the two hydrolases is presumably concerned with the presence of the acetyl group, which causes steric hindrance with the enzyme producing the octasaccharide. This suggests that the acetyl group may occur on the monosaccharide unit adjacent to the fucosyl bond normally attacked, probably the fucose residue, since the acetyl group has been shown to be attached to the tetrasaccharide at the non-reducing end of the octasaccharide. The results with the acetylating system suggest that either the fucose or the glucuronic acid unit is acetylated, and according to the results of Conrad *et al.* (1966) two positions are free from glycosidic bonds on both the glucuronosyl and the fucosyl residues. These are the 2- and 3-positions of the glucuronic acid and the 2- and 4-positions of the fucose. It would thus seem that in the intact polysaccharide one of these four positions is acetylated. The other unidentified component could also be attached to one of the free hydroxyl groups of the fucose, glucuronic acid or glucose. Attempts to identify this component by nuclear-magnetic-resonance spectroscopy of the tetrasaccharide O1 failed to reveal any entity not accounted for by the known structure of the oligosaccharide.

The optimum transfer of [^{14}C]acetyl from [^{14}C]acetyl-CoA to the disaccharide α -glucuronosylfucose may indicate the mode of synthesis of the polysaccharide. No transfer to deacetylated polysaccharide or to non-diffusible material from acid hydrolysis could be shown (I. W. Sutherland, unpublished work). The lack of appreciable transfer

to free fucose or glucuronic acid or to the trisaccharide or octasaccharide units suggests that the acetylating system is acting either on the free disaccharide or on an intermediate in which either a fucose or glucuronic acid on the disaccharide is attached to some other unit. We have been unable to show the occurrence of the free disaccharide or of nucleotide oligosaccharide units as intermediates in biosynthesis, and the acceptor may rather be a monosaccharide-lipid or a disaccharide-lipid complex. Such compounds have been implicated in the biosynthesis of bacterial lipopolysaccharides (Wright, Dankert & Robbins, 1965) and bacterial mucopeptides (Anderson, Mitsuhashi, Haskin & Strominger, 1965). They have recently been identified as precursors of *K. aerogenes* exopolysaccharide (E. C. Heath, personal communication). The only other acetylated polysaccharide from bacteria to be studied in this way is the lipopolysaccharide of *S. anatum* (Robbins *et al.* 1965; Keller, 1966). The system reported differs from that of *K. aerogenes* A3 (Sl) in that enzymic transfer of acetyl groups from acetyl-CoA to either oligosaccharides or complete polysaccharide could be shown. The enzyme used was a particulate preparation and transfer occurred only slowly.

The underlying cause or function of the acetylation of alternate tetrasaccharide units remains obscure. It is difficult to see what advantage it gives the organism over a polysaccharide in which all the tetrasaccharide units are acetylated. At first sight such an octasaccharide repeating unit would seem to require a considerably more complex biosynthetic system than the unacetylated polysaccharide. The additional number of enzymes will depend on their specificity, but the system is likely to be more complex than that in which all the tetrasaccharide units are acetylated.

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