Phage-Induced Fucosidases Hydrolysing the Exopolysaccharide of *Klebsiella aerogenes* Type 54 [A3(SI)]

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Several strains of bacteriophage have been isolated that induce the formation of a polysaccharide hydrolase after infection of *Klebsiella aerogenes* type 54 [A3(SI)]. The action of this enzyme on polysaccharide solutions was to decrease their viscosity and increase their reducing value. These effects were associated with the release of two oligosaccharides (O1 and O2) from the polysaccharide. These two substances are not identical with any of the four oligosaccharides isolated from autohydrolysates. The two enzymically isolated fractions have been tentatively identified as tetrasaccharides, and oligosaccharide O2 is probably an acetylated version of oligosaccharide O1. This latter oligosaccharide differs in some way, still unknown, from the tetrasaccharide cellobiosylglucuronosylfucose found in acid hydrolysates of the slime polysaccharide. The enzyme is limited in its activity to the polysaccharide excreted by the A3 strain of *K. aerogenes* type 54 or by similar strains. It is also active on the polysaccharides altered by acid or alkaline treatment. The enzyme has optimum activity at pH 6.5. A study of the products released by enzyme action has shown it to be a fucosidase splitting the fucosylglucose linkages found in the intact polysaccharide.

The production of enzymes active against capsular polysaccharides is known to be a feature of phage-infected cells of encapsulated bacterial species. Such enzymes may either break down the capsule to permit egress of the mature phage particles, or they may help in the dissolution of the capsules of fresh host cells, thus allowing further phage multiplication. Several such systems have been examined, e.g. the phage-*Klebsiella pneumoniae* B system (Adams & Park, 1956) and the phage-*Azotobacter vinelandii* system (Eklund & Wyss, 1962). Sutherland & Wilkinson (1965) reported the results of studies on the production and mode of action of enzymes on colanic acid after viral infection of *Escherichia coli* K12 and *Aerobacter cloacae* cells. A feature of all these studies was that, although marked decrease in the viscosity of the bacterial polysaccharide solutions occurred, no small-molecular-weight products such as oligosaccharides were isolated. Thus the actual mode of action of these enzymes remains obscure.

The strain of *Klebsiella aerogenes* type 54 [A3(SI)] produces a slime polysaccharide containing the sugars glucose, fucose and glucuronic acid (Wilkinson, Dudman & Aspinall, 1955). The capsular strain from which the A3 variant was derived contained the same sugars in the same proportions as in the slime. Studies on the structure of these

polysaccharides initially suggested that they were extremely complex branched polymers (Aspinall, Jamieson & Wilkinson, 1956). However, re-examination of the slime polysaccharide excreted by *K. aerogenes* A3(SI) indicates that the structure is relatively simple, consisting essentially of a polymer of a four-sugar repeating unit (Sandford & Conrad, 1966; Conrad, Bamburg, Epley & Kindt, 1966).

The present paper reports the isolation of bacteriophage-induced enzymes hydrolysing the *K. aerogenes* A3 polysaccharide to recognizable oligosaccharides and provides supporting evidence for the simplified structure proposed by Conrad and his co-workers. A preliminary account of this work has been presented (Sutherland, 1966).

MATERIALS AND METHODS

Bacteria and bacteriophages. *K. aerogenes* strains A1, A3 and A3(SI), all of serotype 54 and producing polysaccharides of the same chemotype, were those used by Dudman & Wilkinson (1956). They were maintained on nutrient agar slopes in screw-capped vials.

Phages F31, F32, F35, F37 and F38 were isolated independently from sewage, and phages F33, F34 and F36 were isolated from soil, leaf mould and pig faeces respectively. The methods of isolation and of production of stock cultures and of enzymes were those used for *E. coli* bacteriophages by Sutherland & Wilkinson (1965). The enzymes were prepared from phage-infected cultures of either *K. aerogenes*

A3 or A3(SI). A partial purification was achieved by precipitation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained at 40–50% saturation contained all the depolymerase activity. This was used as 'enzyme' after removal of $(\text{NH}_4)_2\text{SO}_4$ by dialysis against 0.02 M-phosphate buffer, pH 7.2.

Production of bacterial polysaccharides. The strains of *Klebsiella* differed in that strains A1 and A3 produced both capsules and slime whereas strain A3(SI) produced slime only. The polysaccharides were prepared from bacterial cultures grown on trays of solid medium (Sutherland & Wilkinson, 1965). The polysaccharides were isolated and purified as described by Dudman & Wilkinson (1956).

Analytical methods. D-Glucose was determined by a micro-adaptation of the glucose-oxidase (β -D-glucose-oxygen oxidoreductase, EC 1.1.3.4) reaction with a commercial enzyme preparation (C. F. Boehringer und Soehne G.m.b.h., Mannheim, Germany). Fucose and glucuronic acid were determined by micro-adaptations of the methods of Dische & Shettles (1948) and of Bowness (1957) respectively. α -Glucosidase (α -D-glucoside glucosylhydrolase, EC 3.2.1.20) was prepared from yeast by the method of Robbins & Uchida (1962). β -Glucosidase (β -D-glucoside glucosylhydrolase, EC 3.2.1.21) and cellulase (β -1,4-glucan glucanohydrolase, EC 3.2.1.4) were purchased from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) and β -glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31) was from British Drug Houses Ltd. (Poole, Dorset).

Paper electrophoresis. The buffer used contained pyridine-acetic acid-water (5:2:43, by vol.). For preparative purposes sheets of Whatman 3MM paper (77 cm. $\times$ 20 cm.) were used, and Whatman no. 1 paper was used for comparative runs, for desalting or for removal of enzyme proteins. Separation of charged oligosaccharides was accomplished by using 80–100 ma for 4–5 hr., and removal of salts or protein was complete in 30–40 min. All electrophoresis was performed on a Locarte high-voltage paper-electrophoresis equipment.

Paper chromatography. The systems used for descending paper chromatography on Whatman no. 1 paper were: 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 48 hr., 20 hr., 24 hr. and 120 hr. respectively. Sugars and oligosaccharides were detected with alkaline AgNO_3 reagent (Trevelyan, Procter & Harrison, 1950).

Borohydride reduction. This was used to ascertain the reducing end-group sugars. To each test tube (75 mm. $\times$ 10 mm.) were added at 0°: 5 μ l. of oligosaccharide solution (approx. 0.5 μ mole), 50 μ l. of water and 10 μ l. of 2.28% (w/v) NaBH_4 in 0.01 N-KOH. The tubes were stoppered and left 12 hr. in the dark at room temperature. Excess of borohydride was destroyed by the addition of 10 μ l. of 2.0 N-acetic acid and salts were removed by electrophoresis before hydrolysis and analysis.

Hydrolysis was performed with 2 N- H_2SO_4 for 4–20 hr. in sealed tubes and was followed by neutralization with Amberlite IRA-410 (HCO_3^- form) resin or with saturated $\text{Ba}(\text{OH})_2$ solution. For all the microchemical and enzymic work, constriction pipettes (H. E. Pedersen, Copenhagen, Denmark) were used. All glassware was thoroughly cleaned with pure HNO_3 and glass-distilled water.

Determination of enzyme activity. Two methods were used. (i) Twofold dilutions of the enzyme were added as 0.02 ml. drops to the surface of 7-day-old cultures of *K. aerogenes* A3(SI) in the same manner as was used with the phage-*E. coli* system by Sutherland & Wilkinson (1965). (ii) Alternatively, the decrease in viscosity of a polysaccharide solution (0.1%, w/v) was measured in an Ostwald viscometer as described in the text.

Buffers. Except where otherwise stated, phosphate buffers (0.05 M) were prepared from KH_2PO_4 and Na_2HPO_4 of A.R. grade.

RESULTS

Exopolysaccharide elaborated by *K. aerogenes* A3(SI). The polysaccharide excreted as a slime into the culture medium by the cells of *K. aerogenes* A3(SI) was isolated and purified as described above. The product resembled that described by Dudman & Wilkinson (1956) and by Sandford & Conrad (1966). Samples hydrolysed with 2 N-sulphuric acid in sealed tubes at 100° for 12 hr., followed by neutralization and chromatography in solvent B, showed the presence of fucose, glucose, glucuronic acid and glucuronolactone. The presence of glucose in the D form was confirmed by tests with glucose oxidase. Some of the polysaccharide (1.0 g.) was dissolved in distilled water and converted into the free acid form by passage through an ion-exchange resin, Amberlite IR-120 (H^+ form). It was then allowed to autohydrolyse in tightly stoppered tubes at 100° for 16 hr. Examination of the autohydrolysate by paper electrophoresis and by paper chromatography in solvents B, C and D showed the presence of neutral material together with three charged fractions. Preparative separation was achieved by paper electrophoresis. This yielded the pure charged fractions. The neutral material was separated into two components by chromatography in solvent A.

Characterization of the fractions obtained by autohydrolysis. In solvent A, the neutral material was resolved into free glucose and an oligosaccharide (N1). No free fucose was observed. Hydrolysates of oligosaccharide N1, when neutralized and chromatographed in solvent B, revealed glucose as the sole constituent sugar. On treatment with borohydride 50% of the glucose was reduced. The enzyme β -glucosidase caused complete hydrolysis of oligosaccharide N1 to glucose, but α -glucosidase was inactive. On paper chromatography in solvents A, B and C, oligosaccharide N1 moved at the same rate as an authentic sample of cellobiose. As further confirmation of its identity with cellobiose, paper electrophoresis in 0.05 M-borate buffer, pH 8.5, at 120 ma for 4 hr. was used. Again oligosaccharide N1 and cellobiose moved at the same rate. From these results it was concluded that oligosaccharide N1 was O- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose.

All three charged fractions moved more slowly in paper electrophoresis than did glucuronic acid.

They were named E1, E2 and E3 in order of increasing mobility.

Fraction E3. Paper chromatography of hydrolysates of this fraction in solvent *B* revealed the presence of fucose and glucuronic acid in approximately equal amounts. This was confirmed by analysis. Treatment with borohydride reduced all the fucose to fucitol. The enzyme β -glucuronidase liberated 28% of the available glucuronic acid. Attempts to increase the amount of enzymic hydrolysis were unsuccessful. As no α -linked glucuronides were available for comparison it was not possible to determine whether the enzyme preparation might contain some contaminating α -glucuronidase. However, it seems probable that fraction E3 is a disaccharide, *O*- β -D-glucuronosyl-fucose.

Fraction E2. This oligosaccharide contained equimolar proportions of fucose, glucose and glucuronic acid. The terminal reducing sugar, as determined by borohydride reduction, was fucose. Hydrolysis with N-sulphuric acid for 15 min. at 100° liberated very small amounts of fucose and glucuronic acid, together with glucose and disaccharide E3. It thus seems probable that fraction E2 is the trisaccharide D-glucopyranosylglucuronosyl-fucose. No free glucuronic acid was liberated on treatment with β -glucuronidase, nor has α -glucosidase or β -glucosidase any effect. The only clue to the nature of the glucosyl linkage was obtained from the use of a cellulase preparation. This was known to be contaminated with one or more β -glucosidases. Treatment of trisaccharide E2 with these enzymes did yield small amounts of disaccharide E3 along with some free glucose. It is therefore probable that the glucose is β -linked to the glucuronic acid.

Fraction E1. The slowest-moving oligosaccharide obtained on autohydrolysis contained glucose, fucose and glucuronic acid in the molar proportions 2:1:1. Borohydride treatment led to reduction of all the fucose. No hydrolysis was obtained with

α -glucosidase, β -glucosidase or β -glucuronidase. Treatment with cellulase led to liberation of 54% of the available glucose. Hydrolysis with N-sulphuric acid at 100° for 15 min. yielded the free sugars comprising the oligosaccharide, together with cellobiose, trisaccharide E2 and disaccharide E3. From these results it would seem that oligosaccharide E1 is a cellobiosyl derivative of disaccharide E3, i.e. *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucosyl-glucuronosylfucose.

The yields of these oligosaccharides together with their properties are shown in Table 1. A comparison of the oligosaccharides obtained on mild acid hydrolysis by Conrad *et al.* (1966) showed them to be identical in all respects when tested with those from autohydrolysis. The oligosaccharides obtained by autohydrolysis indicate that the polysaccharide of *K. aerogenes* A3 (Sl) is a relatively simple structure constructed of a four-sugar repeating unit, a polymer of tetrasaccharide E1.

Specificity of phage enzymes. The phage-induced enzymes from cultures of *K. aerogenes* were unlike the *E. coli*-phage F1 system reported by Sutherland & Wilkinson (1965) as no substrate-controlled induction was observed. The enzymes could also be obtained after growth of bacteriophage on non-exopolysaccharide-producing mutants of *K. aerogenes* A3. As judged by the dissolution of polysaccharide on old lawns of bacteria, or by decrease in the viscosity of 1% (w/v) polysaccharide solutions, the enzymes exhibited a high degree of specificity. Activity was only detected against the polysaccharides produced by *K. aerogenes* A3 (capsule), *K. aerogenes* A3 (Sl) (slime) and *K. aerogenes* strain K66. This last strain, obtained from Dr N. Datta (Lister Institute, London), appeared to be identical with strain A3 with respect to its capsular polysaccharide and to its lipopolysaccharide (Sutherland & Wilkinson, 1966). As it shows the same phage-sensitivity as does strain A3 (D. G. McPhee, unpublished work) it would seem that the two strains are in fact identical. No activity was detected when

Table 1. *Properties of oligosaccharides isolated from autohydrolysates of slime polysaccharide*

GlcUA, Glucuronic acid; Fuc, fucose; M_{GlcUA} , electrophoretic mobility relative to glucuronic acid.

Oligo-saccharide	Yield (μ moles/g. of polysaccharide hydrolysed)	Component sugars	Molar propor- tions	M_{GlcUA}	R_{Glc} in solvent				Hydrolysis (%) by	
					A	B	C	D	β -Gluco- sidase	β -Gluco- ronidase
N1	3.7	Glc	2	—	0.68	0.91	0.34	—	100	—
E1	0.7	Glc, GlcUA, Fuc	2:1:1	0.37	—	—	0.08	0.02	55*	0
E2	1.9	Glc, GlcUA, Fuc	1:1:1	0.51	—	—	0.31	0.25	11*	0
E3	5.2	GlcUA, Fuc	1:1	0.68	—	—	0.67	0.27	—	28

* Cellulase preparations gave hydrolysis, but commercial β -glucosidase (emulsin) was inactive.

the enzymes were tested against the oligosaccharides isolated from autohydrolysates or capsular or slime polysaccharides produced by several other *K. aerogenes* strains, by *Aerobacter cloacae* N.C.T.C. 5920 or by *E. coli* strains. These results were of particular interest because the strains of *K. aerogenes* tested included A1. Analysis of the *K. aerogenes* A1 polysaccharide had shown a composition almost identical with that of *K. aerogenes* A3 polysaccharide (Dudman & Wilkinson, 1956). Both substances contained 9–10% of fucose, 47–48% of glucose and 29–30% of glucuronic acid. The resistance of the *K. aerogenes* A1 polysaccharide to enzymic hydrolysis led us to compare partial acid hydrolysates with those obtained from *K. aerogenes* A3 capsular or slime material: considerable differences were found (S. E. B. Gould & I. W. Sutherland, unpublished work). Thus it is apparent that the similarities in gross composition shown by these two polysaccharides are not reflected in their structure. However, the *K. aerogenes* A3 polysaccharide has recently been reanalysed by Sandford & Conrad (1966) with different methods from the earlier workers: the results obtained, indicating fucose:glucose:glucuronic acid molar proportions 1:2:1, cast doubt on the values reported by Dudman & Wilkinson (1956).

It is probable that, despite their independent isolation, all eight bacteriophages are very similar. This seemed likely from the fact that each enzyme preparation from phage-infected cells produced the same oligosaccharides from the *K. aerogenes* A3 polysaccharide as substrate. Further evidence confirming this was obtained from antiserum-inhibition studies. An antiserum against a preparation of phage F31 enzyme was used. The antiserum (0.1 ml.) was incubated at 37° for 30 min. with a phage-enzyme preparation of titre 1:128. The mixture was then titrated for enzyme activity in the normal way. In all eight preparations the enzyme was completely inhibited by antiserum to phage F31 enzyme. The same result was obtained with antisera to the other enzyme preparations. Similarly phage adsorption and multiplication was inhibited by antisera to any one of the bacteriophages. Despite these similarities some differences were found; thus bacterial mutants resistant to certain of the phages were susceptible to others. Also, polysaccharide hydrolysed by one of the phage enzymes could be hydrolysed further by enzyme from another phage (see below).

Effect of phage enzymes on the bacterial polysaccharide. Preliminary experiments showed that incubation of polysaccharide solutions with the phage enzymes resulted in: (i) an increase in the reducing value; (ii) a decrease in the viscosity.

The reducing value of an aqueous solution of the polysaccharide (5 mg./ml.) was negligible when

Fig. 1. Liberation of reducing material by different polysaccharide hydrolases. A solution of polysaccharide (5 mg./ml.) was incubated with phage F32 enzyme (20 units/ml.) at 37°, and samples were withdrawn and reducing sugar was measured by the method of Somogyi (1945). After 24 hr., the sample was divided into three portions: ●, no further additions; ▲, phage F32 enzyme (10 units/ml.) added; ■, phage F31 enzyme (10 units/ml.) added.

estimated by the method of Somogyi (1945). However, addition of any one of the phage enzymes led to a fairly rapid increase in reducing value, reaching a maximum after about 24 hr. incubation at 30°. The system used involved the addition of 1 ml. of enzyme of titre 1:64 to 20 ml. of polysaccharide solution. Samples were withdrawn at intervals and their reducing values determined. The results of a typical experiment are shown in Fig. 1, where the enzyme used was obtained from cells infected with phage F32. Similar results were obtained with each of the eight enzymes. The addition of further homologous enzyme did not lead to any further increase in the final reducing value. On the other hand, addition of a heterologous enzyme did cause a further release of reducing material. The results of an experiment where enzymic digestion with phage F32 enzyme was followed by addition of phage F31 enzyme are shown in Fig. 1. It is difficult to understand why, if the enzymes from different phage preparations are as similar as the antiserum-inhibition results indicate, further reducing sugar is liberated by heterologous enzyme.

The activity of phage enzymes could also be measured by following the decrease in viscosity of a solution of the substrate. A viscometer was used in which 7.5 ml. of water took 60 sec. to flow through at 37°. A solution of polysaccharide in 0.05 M-phosphate buffer of the appropriate pH was used. Normally 5 ml. of solution containing a total of 1.5 mg. of polysaccharide was used. The enzyme solution to be tested (2.5 ml.) was then warmed to 37°, mixed with the polysaccharide solution in the viscometer and the flow time noted. The final

polysaccharide concentration was thus 1 mg./ml. Readings were continued until the flow time was decreased by 50%.

The results for increasing enzyme concentration are shown in Fig. 2, and those for increasing temperature are shown in Fig. 3. It can be seen that there is a rapid decrease in viscosity with increasing enzyme concentration, leading to a flattening out of the curve. At enzyme concentrations higher than

Fig. 2. Effect of polysaccharide-hydrolase concentration on the viscosity of a polysaccharide solution. The mixture of enzyme and polysaccharide (1 mg./ml.) was incubated at 37° and pH 6.5. The time taken for a halving of the viscosity was determined.

Fig. 3. Effect of temperature on polysaccharide-hydrolase activity as measured by viscosity change. Mixtures of polysaccharide (1 mg./ml.) and enzyme (50 units/ml.) in buffer, pH 6.5, were incubated at different temperatures. The time taken for a halving of the viscosity was determined.

80 units/ml. the decrease in viscosity was so rapid that no meaningful results could be obtained. This was also observed when the enzyme reaction was followed at temperatures above 55°.

In a similar series of experiments, the pH-dependence of the enzymes was measured. The results shown in Fig. 4 clearly indicate the limited range of pH values over which the enzyme proteins retain their activity. This range was pH 5.0–8.0 with an optimum at pH 6.5, a value chosen for all other determinations of enzyme activity and for experiments involving these enzymes. All the enzyme preparations were similar with regard to the effects of pH and of temperature on their activity.

Preparation of enzymic digests of polysaccharide and examination of the products. Although the results quoted here were obtained with the slime polysaccharide, exactly similar results were found with capsular polysaccharide from the parent bacterial strain *K. aerogenes* A3. However, the easier isolation and purification of the slime led to its use. An aqueous 1% (w/v) solution was prepared and 2 ml. of partially purified enzyme solution was added/100 ml. The mixture was stirred gently at 30° for 36 hr. It was then transferred to a dialysis sac and each 100 ml. was dialysed against 500 ml. of distilled water at 0° for 72 hr. This was repeated with fresh distilled water. The two batches of water containing diffusible material were pooled and concentrated under reduced pressure until a thick syrup was obtained. The residual material within the dialysis sac was precipitated with cold acetone in the same manner as was the original polysac-

Fig. 4. Effect of pH on polysaccharide-hydrolase activity as measured by viscosity change. Mixtures of polysaccharide (1 mg./ml.) and enzyme (50 units/ml.) in buffers of various pH values were incubated at 37°. The time taken for a halving of the viscosity was determined.

Table 2. *Properties of the oligosaccharides O1 and O2 isolated from enzymic hydrolysates of slime polysaccharide*GlcUA, Glucuronic acid; Fuc, fucose; M_{GlcUA} , electrophoretic mobility relative to glucuronic acid.

Oligosaccharide	Yield ($\mu\text{moles/g.}$ of poly- saccharide hydrolysed)	Component sugars	Molar proportions	M_{GlcUA}	R_{Glc} in solvent			
					A	B	C	D
O1	220	Glc, GlcUA, Fuc	2:1:1	0.48	0.05	0.49	0.08	0.01
O2	190	Glc, GlcUA, Fuc	2:1:1	0.45	0.07	0.63	0.18	0.03

charide. In typical experiments the residual acetone-precipitable material comprised 10–20% of the original polysaccharide, and analysis showed that it was similar in chemical composition to the slime polysaccharide.

Preliminary experiments indicated that the diffusible material was composed of two components in approximately equal amounts. A number of separation methods were tested, but the only one giving satisfactory separation was preparative paper chromatography with Whatman no. 1 paper (57 cm. $\times$ 46 cm.) irrigated for 24 hr. with solvent B. The syrup was applied as a strip 10 cm. from the end of the paper, and at the end of the run strips 2 cm. wide were cut from each side of the sheet of paper and from the middle. These were stained with alkaline silver nitrate and in each case two oligosaccharides were detected. These had R_{Glc} 0.49 and 0.63 respectively. Occasionally, preparations contained small amounts of material remaining very close to the origin. Attempts to elute this and purify it by other methods were unsuccessful.

The two oligosaccharides were eluted from the paper and the resulting solutions concentrated under reduced pressure, then redissolved in a small volume of water. Chromatography in solvents B, C and D and paper electrophoresis for up to 6 hr. indicated that both fractions were pure. The products from all eight enzyme systems appeared to be identical and they were named oligosaccharide O1 (R_{Glc} 0.49 in solvent B) and oligosaccharide O2 (R_{Glc} 0.63 in solvent B). In all chromatographic systems oligosaccharide O2 moved faster than oligosaccharide O1, but on paper electrophoresis oligosaccharide O1 was the faster-moving (see Table 2).

Hydrolysates of both oligosaccharides (N-sulphuric acid at 100° for 16 hr.), when subjected to paper chromatography in solvent B, showed the presence of glucose, fucose, glucuronic acid and glucuronolactone. In each case, the glucose:fucose:glucuronic acid proportions were 2:1:1 and borohydride reduction indicated that fucose was the terminal reducing sugar in both oligosaccharides. These results indicated that oligosaccharides O1

and O2 were both tetrasaccharides. However, a comparison of the data in Tables 1 and 2 shows clearly that neither oligosaccharide O1 nor oligosaccharide O2 was identical with the tetrasaccharide E1 obtained by autohydrolysis. No hydrolysis of either fraction was detected with α -glucosidase, β -glucosidase, β -glucuronidase or cellulase.

Graded hydrolysis of the two fractions was performed with 0.01N-sulphuric acid at 100° for up to 60 min. The hydrolysates were neutralized with barium hydroxide and examined by electrophoresis and paper chromatography. In oligosaccharide O1, glucuronic acid, disaccharide E3 (glucuronosyl-fucose) and neutral material (fucose, glucose and cellobiose) were detected after several minutes, the amounts of each increasing with time up to 60 min. The results with oligosaccharide O2 were slightly different, as the main disintegration products were aldobiuronic acid, glucose and cellobiose. Very little fucose or glucuronic acid was liberated. In neither case was the trisaccharide E2 or the tetrasaccharide E1 detected. These results indicate that both oligosaccharides were constructed from the two disaccharides cellobiose and glucuronosyl-fucose, most probably in the ratio 1:1. The high chromatographic mobilities in solvent B also indicated that oligosaccharides O1 and O2 were unlikely to be larger than tetrasaccharides. If this is so, some explanation of the differences between oligosaccharides O1, O2 and E1 was necessary. The report by Davies, Crumpton, Macpherson & Hutchison (1958), indicating that the polysaccharide of *K. aerogenes* A3(S1) contained O-acetyl groups amounting to 4% of the dry weight, may offer a possible explanation.

Incubation of oligosaccharides O1 and O2 with aqueous ammonia (1:1, v/v) at 37° for 24 hr. or with 0.01N-sodium hydroxide for 2 hr. at room temperature led to no change in oligosaccharide O1, but converted much of the oligosaccharide O2 into oligosaccharide O1 as determined by electrophoretic and chromatographic mobility. The same result was obtained when the mixtures were heated at 100° for 5–10 min. With 0.1N-sodium hydroxide at

100°, both fractions were hydrolysed to the aldo-biuronic acid and neutral material. Further indication that oligosaccharide O2 was an acylated version of oligosaccharide O1 was obtained by treating the intact polysaccharide with 0.1N-sodium hydroxide at 37° for 24 hr. or with concentrated ammonia under the same conditions. When the polysaccharide was dialysed free of alkali and then treated with a phage-induced depolymerase, no oligosaccharide O2 was detectable in the enzyme digest, only oligosaccharide O1.

On partial autohydrolysis of the slime polysaccharide in the manner used to obtain the E series of oligosaccharides another possible substrate for the enzyme was obtained. The hydrolysis time was shortened to 4 hr. and the diffusible material removed by dialysis against running tap water. The non-diffusible substances were then exposed to enzyme action for 12 hr. Any oligosaccharides released by enzyme action were isolated in the same manner as were oligosaccharides O1 and O2. Only one spot was found on paper electrophoresis or on paper chromatograms. This spot moved at the same rate as did the tetrasaccharide E1, and it too could be hydrolysed to the trisaccharide glucosylglucuronosylfucose by the enzyme cellulase. Autohydrolysis thus removed some grouping that differentiates oligosaccharides O1 and E1. The nature of this grouping remains obscure.

DISCUSSION

Although the induction of enzymes after phage infection of bacteria is a phenomenon that has been quite widely studied, the polysaccharide hydrolases or depolymerases have yielded relatively little information about the mode of action of these enzymes and the structure of their substrates. The only detectable feature has been the dissolution of a polysaccharide gel or the decrease in viscosity of a polysaccharide solution (Adams & Park, 1956; Eklund & Wyss, 1962; Sutherland & Wilkinson, 1965). The reason for the lack of detectable hydrolysis products might be the extreme complexity of the substrate. Thus the enzymes might be endo-depolymerases splitting branch points within the macromolecule, leaving fragments that are still large and complex. The exopolysaccharide slime excreted by *K. aerogenes* A3 (Sl) is now known to be based on a relatively simple structure, which is essentially a repeating unit of four monosaccharides (Conrad *et al.* 1966). The action of the phage-induced enzymes on this polysaccharide could be that of exoenzymes splitting off the repeating units. It is possible that two other phage-induced depolymerases act similarly. A phage having *K. pneumoniae* type 2 as host induced an enzyme causing increased reducing power in the polysaccharide

solutions (Watson, 1966). In our own Laboratory I have recently found a phage-induced enzyme hydrolysing the polysaccharide of *K. aerogenes* strain A4 to monosaccharides and a disaccharide (I. W. Sutherland, unpublished work).

The properties of the polysaccharide hydrolases in the present study appeared to be similar to those of the systems investigated earlier. The optimum pH value of 6.5 and the relative thermostability closely resembled results obtained with enzymes acting on the polysaccharide of *K. pneumoniae* type 2 (Adams & Park, 1956). It was also possible to employ the same methods for partial purification of the enzymes as were used for similar enzymes from infected cells of *E. coli* (Sutherland & Wilkinson, 1965).

Chemical studies have shown that the polysaccharide slime excreted by *K. aerogenes* A3 (Sl) is not the complex highly branched structure originally proposed (Wilkinson *et al.* 1955). The oligosaccharides isolated after autohydrolysis of the polysaccharide were similar to if not identical with those obtained by Conrad *et al.* (1966). These indicated a simple linear structure composed of a four-sugar repeating unit, namely glucosylglucosylglucuronosylfucose, in contrast with the earlier hypothetical branched structure. The only way in which my results differed from those of Conrad *et al.* (1966) was in the finding that the enzyme β -glucuronidase hydrolysed the disaccharide glucuronosylfucose (E2). However, as already suggested, the possible presence of contaminating α -glucuronidase in my enzyme preparations could not be excluded.

The enzyme preparations produced two oligosaccharides thought to be tetrasaccharides, and it seemed probable that one of these, oligosaccharide O2, was an acetylated version of oligosaccharide O1. This would indicate a more complex structure than that proposed by Conrad *et al.* (1966). It is by no means certain that the phage-induced hydrolases were unique in each preparation. More than one enzyme might have been present and might thus have released several products from the polysaccharide. This might also be possible if the polysaccharide possessed branches composed of the same four-sugar unit as the main chain. Alternatively and more probably, as breakage of the fucoside linkage is involved in production of both oligosaccharides, the acylation or other substitution is insufficient to affect the enzyme action and one enzyme only is involved in the production of both oligosaccharides. Supporting evidence for this is seen in the activity of enzyme preparations against alkali- or acid (autohydrolysed)-treated polysaccharide as substrate.

The two oligosaccharides are produced in almost equal amounts. If one is acetylated and contains 1 acetyl group/4 monosaccharide residues, this

would give a calculated acetyl content for the polysaccharide of 3.9%. This value agrees very well with that of 4% found by Davies *et al.* (1958) for the whole polysaccharide. It is therefore possible that the polysaccharide of *K. aerogenes* A3(SI) is indeed a linear molecule consisting of alternate O1 and O2 units. The actual position of the acetyl group in oligosaccharide O2 has not been determined and is rendered exceedingly difficult by the lability of the glucosylglucuronic acid linkage. As oligosaccharide O1 still differs in some way from the tetrasaccharide obtained by autohydrolysis it probably contains some other substitution as yet undetermined.

These results are of considerable interest when the biosynthesis of the polysaccharide is considered. If the mode of synthesis is similar to that found in bacterial lipopolysaccharides, involving sequential addition of sugars from sugar nucleotides (e.g. Edstrom & Heath, 1964), at least four enzymes would be required to synthesize the unacetylated polysaccharide composed of β -glucosyl-(1 $\rightarrow$ 4)-glucosyl- β -glucuronosylfucose units: (i) a fucosyl-transferase, probably with GDP-fucose as donor; (ii) a glucuronosyltransferase, probably with UDP-glucuronic acid as donor; (iii) glucosyltransferase I; (iv) glucosyltransferase II. In addition, an acetylase would also be involved, acting either at the nucleoside diphosphate sugar level or at the polysaccharide level. A further enzyme would be required for the other substitution thought to exist in oligosaccharides O1 and O2. It is probable that the fucose and glucuronic acid are not constituents of any other cell structures, as they are known to be absent from the lipopolysaccharide of *K. aerogenes* A3(SI) (Sutherland & Wilkinson, 1966). However, the glucose is obviously involved in other cell reactions and control of its incorporation into the exopolysaccharide may be due to utilization of distinct nucleotide donors. Consequently loss of any one of a number of enzymes by mutation might lead to loss of the ability to synthesize the exopolysaccharide. From these considerations and the partial elucidation of the polysaccharide structure,

a clearer idea of the 'mucoid' to 'non-mucoid' mutation should be obtained.

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