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THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* SEROTYPE K54; LOCATION OF THE *O*-ACYL GROUPS, AND A REVISED STRUCTURE

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ABSTRACT

The capsular polysaccharide from *Klebsiella* type K54, containing both *O*-formyl and *O*-acetyl groups, has been investigated by using the techniques of methylation analysis (by gas–liquid chromatography), periodate oxidation–Smith degradation, and both ^{1}H- and ^{13}C-n.m.r. spectroscopy. Degradation of the native polysaccharide with a bacteriophage-induced glucosidase generated a formylated, as well as a formylated and acetylated, tetrasaccharide, whereas similar depolymerization of the deacetylated polysaccharide yielded a single tetrasaccharide; the corresponding, *O*-acylated octasaccharides were also isolated and characterized. These oligosaccharides, utilized in chemical and spectroscopic studies in order to determine the location of the *O*-acyl substituents in the repeating sequence, indicated formylation at O-4 of each lateral D-glucosyl group and acetylation at O-2 of alternate L-fucosyl residues. A new structure for the repeating unit in the polysaccharide is proposed.

INTRODUCTION

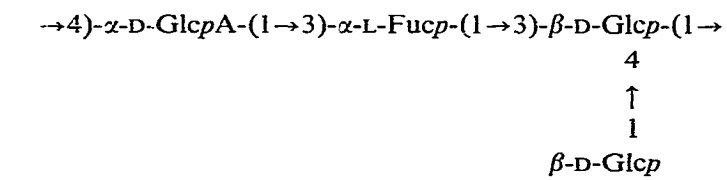
The exopolysaccharide of *Klebsiella* serotype K54 consists of a regular, tetrasaccharide repeating-sequence in which formic and acetic esters have also been identified[1]. Originally believed to have a very complex, nonrepeating structure[2], the chemical repeating-unit of the K54 polysaccharide was determined in 1966, by Sandford and Conrad[3], who re-examined the polymer by using a much improved, methylation methodology. The presence of a branched-tetrasaccharide repeating-sequence has since been well established in subsequent studies on this polysaccharide by using the methods of partial hydrolysis[4,5], gel-permeation chromatography[4], and hydrolysis with bacteriophage-borne enzymes[6,7].

In the last investigation, depolymerization of the K54 polysaccharide with a series of fucosidases considered to be phage-induced allowed the isolation of the intact repeating-unit bearing both *O*-formyl and *O*-acetyl substituents. The characterization of an octasaccharide containing formate and acetate in the ratio of 2:1 indicated that every second tetrasaccharide sequence was *O*-acetylated, and suggested that the structure of the polysaccharide was based on an octasaccharide repeating-unit. The extent of *O*-acetyl substitution was subsequently confirmed by p.m.r.

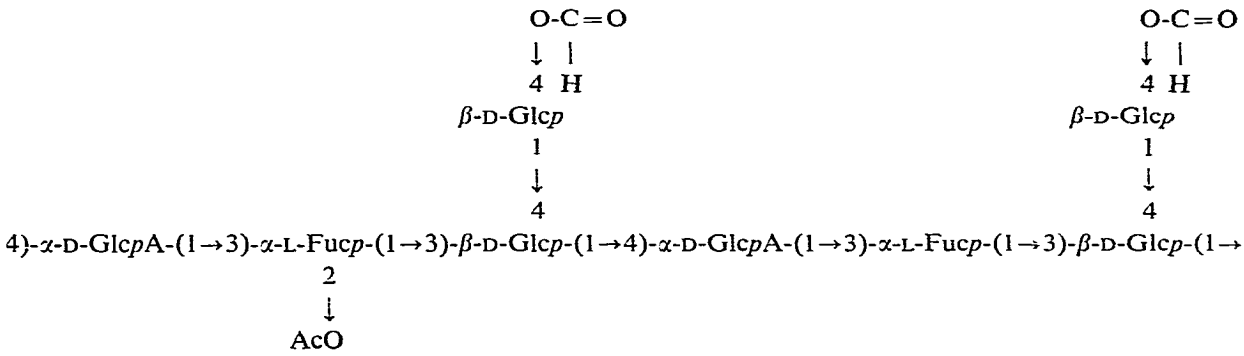
spectroscopy of the K54 polysaccharide[4], but the detection and quantitation of formate by spectroscopic methods has only recently been established. Attempts to determine the sites of *O*-acylation in the repeating sequence have hitherto proved difficult, due to the limitations of the available techniques and also to the susceptibility of these substituents to hydrolysis with acidic and alkaline reagents.

We have recently utilized[8,9] a number of more-modern techniques, including high-resolution, ^{1}H- and ^{13}C-n.m.r. spectroscopy, to locate the *O*-acyl substituents in the capsular polysaccharide from *Klebsiella* K63, and have since applied these methods to the oligosaccharides generated by bacteriophage action on the K54 polysaccharide. In the present study, both the native and deacetylated K54 polysaccharides were depolymerized by using the same bacteriophage-induced glucosidase, and the resulting tetra- and octa-saccharide products were analyzed by standard procedures.

During this investigation, however, it became apparent that the structures of the aforementioned oligosaccharides were inconsistent with those expected on the basis of the proposed, chemical repeating-unit in the K54 polysaccharide. This was particularly evident from ^{13}C-n.m.r. studies on the poly- and oligo-saccharides, which showed two signals in the region of 60–67 p.p.m. attributed to non-linked, primary alcohol groups in D-glucose residues[10], indicating that O-6 of both the terminal D-glucosyl group and the in-chain D-glucosyl residues were unsubstituted. On the basis of these observations, it was considered necessary to re-examine the K54 polysaccharide by using the more recently developed techniques of methylation analysis by gas–liquid chromatography, g.l.c.–m.s., and high-resolution, ^{1}H- and ^{13}C-n.m.r. spectroscopy. From the results obtained, it is evident that the in-chain D-glucosyl residue is linked through O-3, not O-6, in the repeating sequence, giving rise to the primary structure shown.



Methylation and spectroscopic studies on both the native and deacetylated polysaccharides, as well as on the products of bacteriophage degradation, indicated the location of formate at C-4 of the terminal D-glucosyl group in each of the foregoing units, and acetylation at O-2 of every second L-fucosyl residue in the repeating sequence, thus confirming the presence of an octasaccharide repeating-unit in the *Klebsiella* K54 polysaccharide as shown.



Since the work described herein was completed, we have been informed[11] that Albersheim and his colleagues have independently, and by completely different methods, confirmed this revised structure for the K54 polysaccharide.

RESULTS AND DISCUSSION

In order to rationalize the results obtained for the products of bacteriophage degradation, it was necessary to perform certain preliminary analyses on the K54 polysaccharide so as to establish the nature of the chemical repeating-unit in this polymer. These results are presented in Section A, and the characterization of the oligosaccharides generated by bacteriophage-induced depolymerization of the K54 polysaccharide is discussed in Section B.

A. Structure of the K54 polysaccharide; location of formate

N.m.r. spectroscopy; identification of O*-acyl substituents.* — The presence both of *O*-formyl and *O*-acetyl groups in the slime polysaccharide from *Klebsiella aerogenes* type 54 [A3 (51)] was demonstrated by Sutherland[1], who identified these esters by paper chromatography of their derived hydroxamic acids. In the present study, these substituents were characterized by high-resolution, ^{1}H-n.m.r. spectroscopy of a sample of the same polysaccharide, supplied by courtesy of Dr. I. W. Sutherland, Edinburgh. The methyl group of *O*-acetyl gave a sharp singlet at δ 1.92 in the p.m.r. spectrum of the native polysaccharide, but was not present in that of the polysaccharide obtained by treatment with 0.01M potassium hydroxide at room temperature. The signal due to the proton of *O*-formyl occurred as a broad singlet at δ 4.72 in both of these spectra. The assignments for these and other protons in the spectra of both the native and deacetylated K54 polysaccharides are shown in Table I. From the proportions of acetate and L-fucose found on integration of the signals given by the native polysaccharide, it is evident that *O*-acetylation occurs on every second tetrasaccharide repeating-sequence.

De-esterification of the formic esters to yield the corresponding, nonacylated polysaccharide was achieved by treatment of the deacetylated polysaccharide with

TABLE I

N.M.R. DATA FOR THE NATIVE AND *O*-DEACETYLATED *Klebsiella* K54 POLYSACCHARIDE

Polysaccharide	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	δ[a]	$J_{1,2}$ *(Hz)*[b]	*Integral* (H)	*Assignment*	*p.p.m.*[c]	*Assignment*
Native	5.46	b	1.0	α-Fuc		
	5.25	b	1.0	α-GlcA		
	4.74	b	1.0	*O*-formyl		
	4.55	b	1.0	β-Glc (c)[d]	Samples too viscous to provide a good spectrum	
	4.49	b	1.0	β-Glc (t)[d]		
	4.30	b	1.0	H-5 α-GlcA		
	1.92	s	0.5	CH_3 of *O*-acetyl		
	1.19	b	3.0	CH_3 of α-Fuc		
O-Deacetylated	5.45	s	1.0	α-Fuc	173.9	CO of *O*-formyl
	5.27	s	1.0	α-GlcA	102.7	β-Glc (c)[d], β-Glc (t)
	4.75	s	0.8	*O*-formyl	102.0	α-GlcA
	4.55	8.0	1.0	β-Glc (c)	99.5	α-Fuc
	4.49	8.0	1.0	β-Glc (t)	68.0	C-2 of α-Fuc
	4.38	8.0	1.0	H-5 α-GlcA	67.0	C-5 of α-Fuc
	1.20	6.0 ($J_{5,6}$)	3.0	CH_3 of α-Fuc	62.9	C-6 of β-Glc (c)
					61.4	C-6 of β-Glc (t)[e]
					60.5	
					16.1	C-6 of α-Fuc

[a]Chemical shift relative to internal acetone, δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [b]b = broad, unable to assign accurate coupling-constants; s = singlet. [c]Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [d](c) = in-chain, (t) = terminal. [e]Two signals due to the removal of some formate during *O*-deacetylation.

0.1M potassium hydroxide on a steam bath for 18 h. Owing to the high viscosity of solutions of the native polymer in D_2O, a satisfactory ^{13}C-n.m.r. spectrum could not be obtained. The removal of *O*-acetyl, however, is accompanied by a large decrease in the viscosity, and a reasonable ^{13}C-n.m.r. spectrum of this material was obtained, the assignments for which are shown in Table I. The presence of three signals corresponding to nonlinked C-6 atoms of hexose residues (60–63 p.p.m.) gave the first indication that O-6 of both residues of D-glucose in the K54 polysaccharide are unsubstituted. The presence of *three* signals was subsequently explained by the observation that the terminal glucose residue gives two distinct signals for C-6, depending on whether O-4 is or is not formylated. Although no direct evidence for the presence of formate can be obtained from ^{13}C-n.m.r. spectroscopy, the strong carbonyl absorption at 173 p.p.m. is attributed to this ester.

Methylation analysis; location of O*-formyl.* — In three separate experiments, samples of the *O*-formylated polysaccharide were dissolved in dimethyl sulfoxide, stirred with methylsulfinyl anion for 50, 33, and 10 sec, respectively, and then methylated according to the procedures described previously[9]. The fully methylated deriv-

TABLE II

METHYLATION ANALYSES OF THE *O*-FORMYLATED AND THE NONACYLATED *Klebsiella* K54 POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	T[b]	*Molar proportions (mole %)*[c]					
		I[d,e]	*II*[e]	*III*[f]	*IV*[g]	*V*[d,e]	*VI*[d,e]
2,3,4,6-Glc	1.00	36.4	27.7	30.3	35.6	39.8	27.2
2,4-Fuc	1.18	18.6[h]	18.5	18.9	24.2	21.4[h]	21.0
2,3,6-Glc	1.90	10.9	8.2	5.7	3.8	—	—
2,6-Glc	2.47	33.1	24.8	25.3	24.6	38.8	27.8
2,3-Glc	3.08	—	20.8	19.8	21.8	—	24.0

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucose. [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on column *A*. [c]Values corrected by use of the e.c.r. factors given by Albersheim *et al.*[12]. [d]Key: I, methylated, *O*-formylated polysaccharide; II–IV, methylated and reduced, *O*-formylated polysaccharide; V, methylated, nonacylated polysaccharide; VI, methylated and reduced, nonacylated polysaccharide. [e–g]Exposed to methylsulfinyl anion for 10, 33, and 50 s, respectively. [h]Low proportions, due to incomplete hydrolysis of aldobiouronic acid linkages.

atives (as indicated by i.r. spectroscopy and g.l.c. analysis) were carboxyl-reduced with lithium aluminum hydride, the products hydrolyzed, and the sugars present in the hydrolyzates analyzed by g.l.c. of the derived alditol acetates, to give the proportions shown in Table II. (Methylation analysis of the nonreduced polymer after methylation for 10 sec is shown in Column I.) The positions of methoxyl substitution in each methylated alditol acetate were confirmed by g.l.c.–m.s. A sample of the nonacylated polysaccharide was also methylated for 10 sec, carboxyl-reduced, and the product analyzed in the same way (see Table II, columns V and VI).

From the results obtained, it is evident that residues of both 2,3- and 2,6-di-*O*-methyl-D-glucose are present in the hydrolyzates of the methylated and carboxyl-reduced polysaccharides. Methylation analysis of the fully methylated polysaccharide before reduction gave only 2,6-di-*O*-methyl-D-glucose, and this indicates that the in-chain residues of D-glucose in the polysaccharide are substituted at both O-3 and O-4. The proportions of 2,3-di-*O*-methyl-D-glucose found in the aforementioned hydrolyzates following carboxyl reduction of the methylated polymers must, therefore, be due to reduction of the D-glucuronic acid residues, which are linked through O-4 in the repeating sequence. According to the structure proposed from earlier methylation studies[3], we should expect that 2,3-di-*O*-methyl-D-glucose would be present in the hydrolyzate of the methylated polysaccharide, and that the proportion of this sugar would increase markedly in the hydrolyzate of the carboxyl-reduced derivative; that this is clearly not the case may be seen from Table II.

The proportions of 2,3,6-tri-*O*-methyl-D-glucose found in the hydrolyzates of both the methylated and the methylated, carboxyl-reduced polysaccharides are attributed to substitution at O-4 of the terminal D-glucosyl groups with *O*-formyl. The decrease in the proportion of 2,3,6-trimethylglucose formed as the period of

time of exposure of the polysaccharide to the methylsulfinyl anion was increased, and the absence of this methylated sugar in the hydrolyzate of the nonformylated polysaccharide, provide further evidence for the location of the formyl group at O-4 of the terminal D-glucosyl groups in the repeating sequence. Spectroscopic studies performed on the oligosaccharides obtained by bacteriophage-induced depolymerization of the K54 polysaccharide substantiated this assignment (see Section B). The stability of *O*-formyl groups to alkaline reagents, reported by Sutherland[7], is consistent with the results obtained for the capsular polysaccharide from *Klebsiella* K63, which also contains[8] *O*-formyl groups. It should, however, be noted that, although the qualitative data for the methylation of the formylated polysaccharide (see Table II, column I) are satisfactory, there is a discrepancy in the quantitative figures. The sum of the amount of 2,3,4,6-tetra- and 2,3,6-tri-*O*-methylglucose should be equal to that of the 2,6-di-*O*-methyl derivative. Although there was no evidence for undermethylation, it is likely that the branch-point D-glucosyl residue is relatively sterically hindered, thus giving a low value.

Periodate oxidation. — The native and the deacetylated, capsular polysaccharides were oxidized with sodium periodate, and the products were reduced with sodium borohydride. Hydrolysis of the derived polyalcohols, and paper chromatography of the hydrolyzates, in each case showed the presence of glycerol, erythritol, fucose, and glucose. A sample of each hydrolyzate was reduced with sodium borohydride, the alditols were acetylated, and the esters analyzed by g.l.c., to yield the proportions of alditol acetates shown in Table III. From these results, it is clear that the terminal D-glucosyl group is substituted at O-4, thereby giving rise to the high proportions of erythritol found in these hydrolyzates. The presence of a small proportion of glycerol in each is attributed to the removal of some of the *O*-formyl groups during the isolation of the polysaccharide, or during *O*-deacetylation. Furthermore, the presence of L-fucose and D-glucose in equimolar proportions is consistent with the results of the methylation analysis obtained earlier, and is in accord with the revised structure of

TABLE III

ANALYSIS OF THE POLYALCOHOLS DERIVED FROM THE NATIVE AND *O*-DEACETYLATED K54 POLYSACCHARIDE

Peracetylated alditol	T[a]	*Molar proportions (mole %)*[b]	
		Native polysaccharide	*O-Deacetylated polysaccharide*
Glycerol	0.14	9.0	14.6[c]
Erythritol	0.40	28.8	23.3
L-Fucitol	0.70	28.0	30.7
D-Glucitol	1.00	34.0	31.2

[a]Retention time relative to that of D-glucitol hexaacetate on column *B*. [b]Values corrected by use of molar-response factors. [c]Increase in proportion of glycerol due to saponification of formate during *O*-deacetylation.

the K54 polysaccharide. No D-glucuronic acid was detected in the hydrolyzates of the periodate-oxidized products, indicating cleavage between C-2 and C-3 of these residues, and this therefore eliminates O-2 and O-3 as possible sites for acetylation.

Smith hydrolysis, with M trifluoroacetic acid at room temperature, of the polyalcohol derived from the native polysaccharide yielded glycolaldehyde, glycerol, erythritol, and a slower-moving component which was isolated by preparative paper-chromatography, and then methylated. Hydrolysis of the methylated product, and paper chromatography of the hydrolyzate, gave 2,4,6-tri-*O*-methylglucose and 2,4-di-*O*-methylfucose, which, by g.l.c. of their derived alditol acetates, were shown to be present in equimolar proportions. The identification of 2,4,6-tri-*O*-methyl-D-glucose clearly indicates that these in-chain residues of D-glucose are linked at O-3, and that the terminal D-glucosyl group is attached to O-4, as previously shown by the isolation of cellobiose from partial hydrolyzates of the K54 polysaccharide[5,6]. The presence of 2,4-di-*O*-methyl-L-fucose, and not the 2,3,4-tri-*O*-methyl derivative expected, is consistent with the acid acetals's remaining unhydrolyzed under the conditions used for the Smith hydrolysis. This type of behavior has been reported in other studies involving acidic acetals of this nature[13].

B. Depolymerization with bacteriophage-borne enzymes

Isolation and characterization of oligosaccharides. — The capsular polysaccharide from *Klebsiella* K54 was depolymerized with the host bacteriophage ϕ54 according to the methods described earlier[14], to yield the *O*-formylated tetrasaccharide **1**, the *O*-formylated and *O*-acetylated tetrasaccharide **2**, and an octasaccharide **3**. Depolymerization of the deacetylated K54 polysaccharide under similar conditions gave the tetrasaccharide **1**, and the corresponding *O*-formylated octasaccharide **4**. The proportions of formate and acetate in these oligosaccharides were determined by p.m.r. spectroscopy, and the degree of polymerization and the reducing sugar (D-glucose) in each oligomer were established according to the method of Morrison[15]. The results obtained from these and other analyses are summarized in Table IV.

TABLE IV

CHARACTERIZATION OF THE OLIGOSACCHARIDES OBTAINED BY BACTERIOPHAGE-INDUCED DEPOLYMERIZATION OF THE NATIVE AND *O*-FORMYLATED *Klebsiella* K54 POLYSACCHARIDES

Oligosaccharide[a]	$[\alpha]_D$ *(degrees)*	R_{Gal}[b]	*Mol. wt.*[c]	$\overline{DP}$[d]	*Acetate (molar proportions)*[e]	*Formate (molar proportions)*[e]
1	+1	0.12	760	4.0	—	1.0
2	−5	0.23	760	4.0	1.0	1.0
3	−10	0.00	1450	8.0	1.0	2.0
4	−12	0.02	1450	8.0	—	2.0

[a]Glucose was found to be the reducing sugar in each oligomer. [b]Solvent *B*. [c]By gel-permeation chromatography on a column of Bio-Gel P-10. [d]Determined by the method of Morrison[15]. [e]By p.m.r. spectroscopy.

TABLE V

METHYLATION ANALYSES OF THE METHYLATED, AND METHYLATED CARBOXYL-REDUCED, OLIGOSACCHARIDES **1** AND **4**

Methylated sugars[a] *(as alditol acetates)*	T[b]	*Molar proportions (mole %)*[c]			
		I[d,e]	*II*	*III*[f]	*IV*
2,3,4,6-Glc	**1.00**	44.5	29.1	37.2	24.6
2,4-Fuc	1.18	15.0[g]	19.0	14.9[g]	22.4
2,3,6-Glc	1.90	6.3	26.4	3.9	11.8
2,3,4-Glc	1.90	—		—	
2,6-Glc	2.47	34.0	25.5	44.0	27.3
2,3-Glc	3.08	—	—	—	13.9

[a–c]As for Table II. [d]Key: I, methylated tetrasaccharide **1**; II, methylated and reduced tetrasaccharide **1**; III, methylated octasaccharide **4**; IV, methylated and reduced octasaccharide **4**. [e,f]Exposed to methylsulfinyl anion for 25 and 50 s, respectively. [g]Low proportions, due to incomplete hydrolysis of aldobiouronic acid linkages.

Methylation of tetrasaccharide **1** (25 sec in the base), followed by hydrolysis and paper chromatography of the hydrolyzate showed the presence of 2,3,4,6-tetra-*O*-methylglucose, 2,6-di-*O*-methylglucose, 2,4-di-*O*-methylfucose, and a small amount of 2,3,6-tri-*O*-methylglucose. These sugars were quantitatively analyzed by g.l.c. of the partially methylated alditol acetates, as shown in Table V, column I. Carboxyl-reduction of the methylated tetrasaccharide, hydrolysis, and analysis by paper chromatography and by g.l.c. of the sugars present in the hydrolyzate gave the molar proportions shown in Table V, column II. Similar, methylation analysis of the methylated, and methylated carboxyl-reduced, octasaccharide **4** gave the results shown in Table V, columns III and IV. These methylation analyses accord with the structures assigned and with the structure of the repeating sequence in the K54 polysaccharide. The small amounts of 2,3,6-tri-*O*-methylglucose detected in the hydrolyzates of the methylated derivatives are again due to incomplete saponification of the formic esters during the short treatment with base.

^1H- and ^{13}C-N.m.r. spectroscopy; location of the O*-acetyl groups.* — Oligosaccharides **1–4** were examined by high-resolution, p.m.r. spectroscopy, which permitted the proton assignments listed in Table VI. In addition, the tetrasaccharides **1** and **2** were analyzed by ^{13}C-n.m.r. spectroscopy, to give the chemical-shift data (also shown in Table VI). The results of these experiments not only provided further evidence for the location of the formyl group at O-4 of the D-glucosyl groups, but also indicated that the acetyl group in tetrasaccharide **2** is situated at O-2 of L-fucose. These observations are well illustrated by comparison of the ^{13}C-n.m.r. spectra of the two tetrasaccharides (**1** and **2**), as shown in Fig. 1, from which it may be seen that the signal at 68 p.p.m. attributed to C-2 of the α-L-fucosyl residue in tetrasaccharide **1** is shifted downfield by ~2.5 p.p.m. upon *O*-acetylation to yield tetra-

TABLE VI

N.M.R. DATA FOR THE TETRA- AND OCTA-SACCHARIDES OBTAINED BY BACTERIOPHAGE DEGRADATION OF THE NATIVE AND *O*-FORMYLATED *Klebsiella* K54 POLYSACCHARIDES

Oligosaccharide	^{1}*H-N.m.r. data*				^{13}*C-N.m.r. data*	
	δ^a	$J_{1,2}$ (Hz)^b	*Integral* (H)	*Assignment*	*p.p.m.*^{c}	*Assignment*
1	5.44	3.5	0.6	α-Fuc	102.1	β-Glc
	5.38	3.5	0.4		101.5	α-GlcA
	5.26	3.5	1.0	α-GlcA	99.8	α-Fuc
	5.19	3.5	0.4	α-Glc~OH	99.7	
	4.72	6.0	1.0	*O*-formyl	96.7	β-Glc~OH
	4.65	8.0	0.6	β-Glc~OH	92.8	α-Glc~OH
	4.49	8.0	1.0	β-Glc	68.0	C-2 α-Fuc
	4.22	10.0	1.0	H-5 α-GlcA	67.1	C-5 α-Fuc
	1.19	6.0 ($J_{5,6}$)	3.0	CH_3 of α-Fuc	62.9	C-6 β-Glc~OH
					60.5	C-6 β-Glc
					16.1	CH_3 of α-Fuc
2	5.50	3.5	0.3	α-Fuc	102.1	β-Glc
	5.44	3.5	0.3		101.7	α-GlcA
	5.20	3.5	1.0	α-GlcA	97.2	α-Fuc
	5.16	3.5	0.4	α-Glc~OH	96.9	β-Glc~OH
	5.13	3.5	0.2	α-Fuc	92.9	α-Glc~OH
	5.10	3.5	0.2		70.6	C-2 α-Fuc
	4.72	6.0	1.0	*O*-formyl	67.3	C-5 α-Fuc
	4.61	8.0	0.6	β-Glc OH	63.0	C-6 α,β-Glc~OH
	4.51	8.0	1.0	β-Glc	60.5	C-6 β-Glc
	4.24	10.0	1.0	H-5 α-GlcA	21.1	CH_3 of *O*-acetyl
	2.17	s	3.0	CH_3 of *O*-acetyl	16.0	C-6 of α-Fuc
	2.16	s				
	1.21	6.0 ($J_{5,6}$)	3.0	CH_3 of α-Fuc		
3	5.44	3.5	2.0	α-Fuc		
	5.26	3.5	1.0	α-GlcA		
	5.24	3.5	1.0	α-GlcA		
	5.19	3.5	0.4	α-Glc~OH		
	4.74	6.0	2.0	*O*-formyl		
	4.65	8.0	0.6	β-Glc~OH		
	4.54	8.0	1.0	β-Glc (c)		
	4.49	8.0	1.0	β-Glc (t)		
	4.48	8.0	2.0	β-Glc (t)		
	4.27	10.0	1.0	H-5 α-GlcA		
	4.20	10.0	1.0	H-5 α-GlcA		
	2.17	s	3.0	CH_3 of *O*-acetyl		
	2.15	s				
	1.19	6.0 ($J_{5,6}$)	6.0	CH_3 of α-Fuc		

TABLE VI *(continued)*

Oligosaccharide	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	δ^a	$J_{1,2}$ (Hz)[b]	*Integral* (H)	*Assignment*	*p.p.m.*[c]	*Assignment*
4	5.44	3.5	1.6	} α-Fuc		
	5.40	3.5	0.4	}		
	5.28	3.5	2.0	α-GlcA		
	5.19	3.5	0.4	α-Glc~OH		
	4.74	6.0	2.0	*O*-formyl		
	4.66	8.0	0.6	β-Glc~OH		
	4.55	8.0	1.0	α-Glc (c)		
	4.49	8.0	2.0	α-Glc (t)		
	4.39	10.0	1.0	H-5 α-GlcA		
	4.31	10.0	1.0	H-5 α-GlcA		
	1.19	6.0 ($J_{5,6}$)	6.0	CH_3 of α-Fuc		

[a–c]As for Table I.

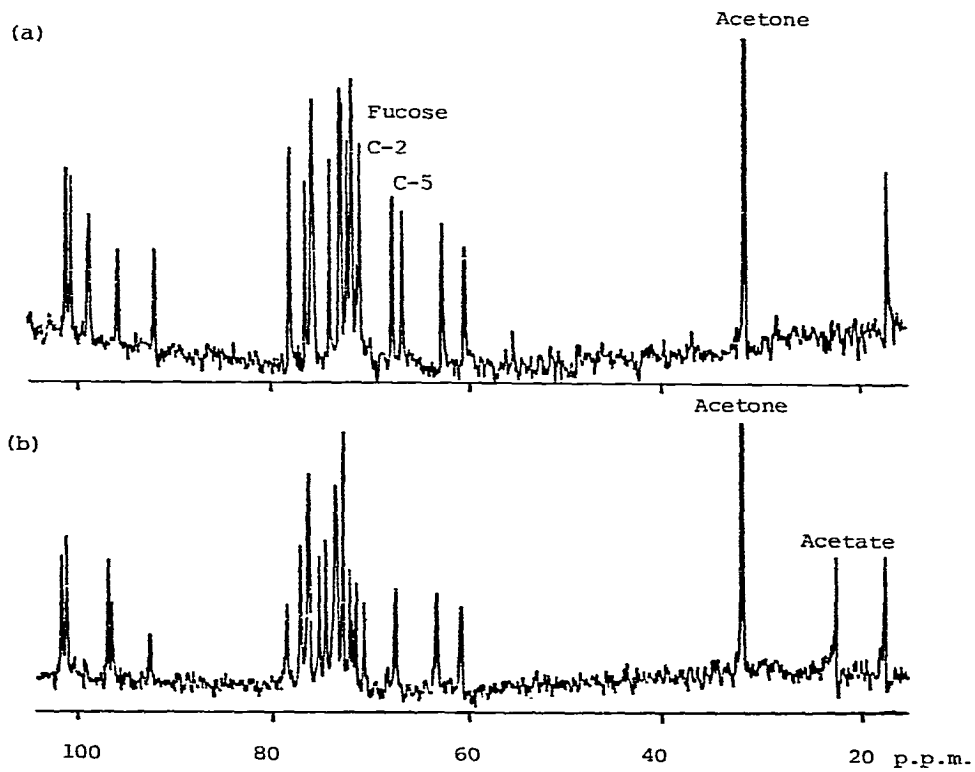


Fig. 1. ^{13}C-N.m.r. spectra of (a) formylated (**1**), and (b) formylated and acetylated (**2**) tetrasaccharides from *Klebsiella* K54 polysaccharide.

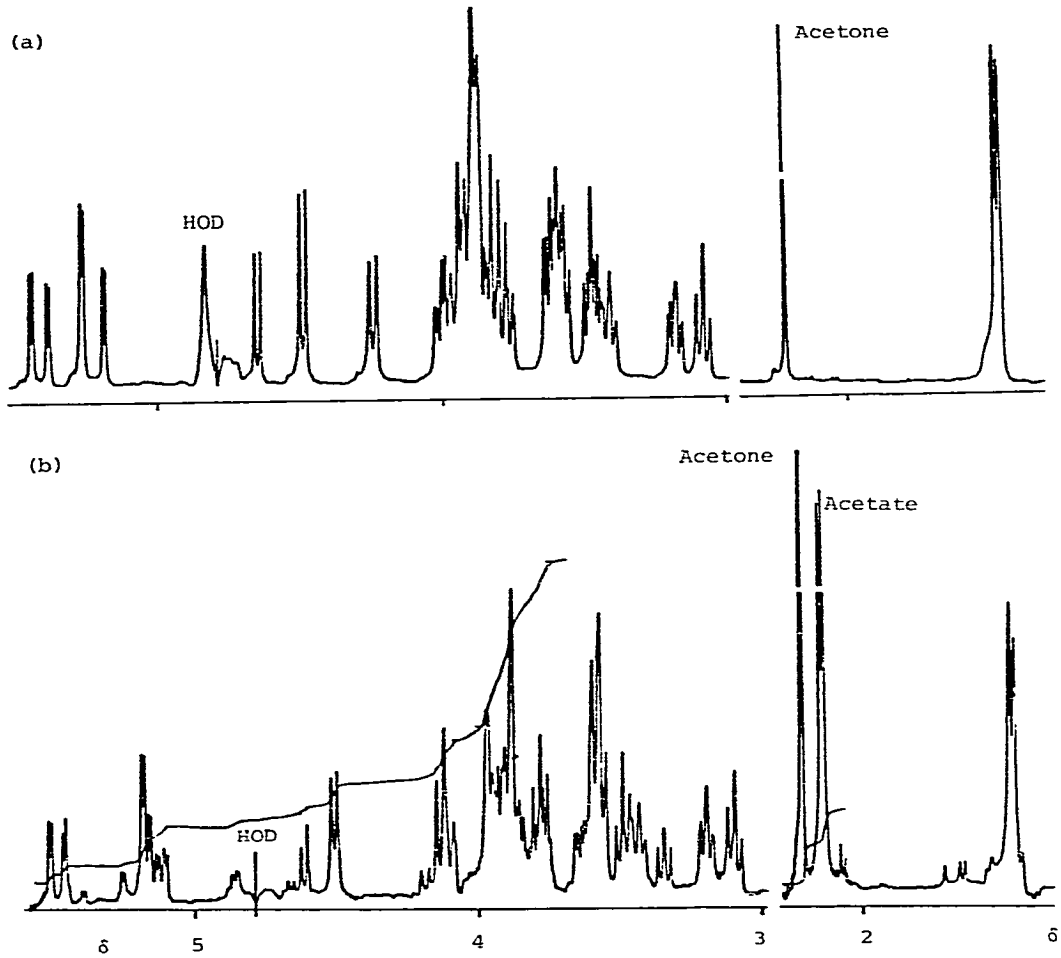


Fig. 2. ^{1}H-N.m.r. (400 MHz) spectra of (a) formylated (**1**), and (b) formylated and acetylated (**2**) tetrasaccharides from *Klebsiella* K54 polysaccharide.

saccharide **2**: the signal due to C-1 of the L-fucosyl residues is also affected by the changes at C-2, and shows a shift to higher field of ~1 p.p.m. Further evidence for acetylation at O-2 of L-fucose was provided by p.m.r. spectroscopy of tetrasaccharides **1** and **2**, which indicated a shift to lower field of the signals due to H-1, H-2, and H-3 of the 6-deoxy sugar in the spectrum of the latter oligosaccharide. Interestingly, the H-1 signal of L-fucose occurs as a pair of doublets in the spectrum of tetrasaccharide **1** [see Fig. 2 (a)], the proportions of which correspond with the anomeric ratio of the (reducing) D-glucose residue. The 400-MHz spectrum of tetrasaccharide **2** [Fig. 2 (b)] shows two sets of doublets in the anomeric region, with fairly large differences in chemical shift, both of which are assigned to H-1 of the α-L-fucosyl residue. The origin of this difference in chemical environment is believed to be due to restricted rotation of the acetyl substituent at O-2, which can result in either the methyl, or

the carbonyl group of the ester being in close proximity to the proton on C-1. The methyl group of acetate occurs as two distinct singlets in the p.m.r. spectra of oligosaccharides **2** and **3**, thus providing further evidence in support of the foregoing proposals.

Substitution of the terminal D-glucosyl group with an *O*-formyl group results in a downfield shift of the resonance due to C-4, but the actual magnitude of the change is difficult to determine, owing to the complexity of the ^{13}C-n.m.r. spectra of tetrasaccharides **1** and **2**. The chemical shift observed for C-4 in (terminal) β-D-glucosyl groups ranges[10] between 70.5 and 71.2 p.p.m.; the ^{13}C-n.m.r. spectrum of tetrasaccharide **1** shows no signals in this region, and the resonance at 70.6 p.p.m. in the spectrum of the acetylated tetrasaccharide **2** was assigned to C-2 of the α-L-fucosyl residue.

The *O*-formyl proton in the spectrum of tetrasaccharide **1** occurs at δ 4.72, and was assigned from the high-resolution, p.m.r. spectrum of this oligosaccharide recorded at high temperature: in spectra recorded at ambient temperature, this signal is obscured by the residual HOD peak. The H-1 resonances assigned to the β-glucosyl group and the (reducing) β-glucose residue are seen at slightly higher field in the p.m.r. spectra of oligosaccharides **1** and **2** [see Fig. 2 (a) and (b)]. The well defined doublet at δ ~4.20 ($J_{4,5}$ ~10 Hz) was assigned to H-5 of the α-D-glucosyluronic residues in these oligosaccharides, and the p.m.r. spectra of octasaccharides **3** and **4** gave two signals in this region, due to the presence both of terminal and in-chain glucosyluronic residues.

The effect of formylation at O-4 of the (terminal) D-glucosyl group is uncertain, as the corresponding, nonacylated tetrasaccharide was not isolated. The p.m.r. spectra of both tetrasaccharides **1** and **2**, however, show at high field two triplets which are well resolved from one another and from the rest of the ring protons. The triplet at δ ~3.20 was unambiguously assigned to H-2 of the (terminal) β-D-glucosyl group by selective decoupling of the corresponding H-1 resonance; similar experiments performed on the triplet at δ ~3.10 indicated that this signal was due to H-4 of the same D-glucosyl group. The chemical shift of this proton is ~25 Hz upfield from that observed for H-4 in the spectrum of methyl β-D-glucopyranoside[16], and is attributed to the presence of a formyl group on O-4 of these D-glucosyl groups in the polysaccharide repeating-unit.

CONCLUSION

The application of modern instrumental techniques to studies of polysaccharide structures has greatly facilitated such investigations. The use of these methods in conjunction with specific bacteriophage-borne enzymes promises to become a powerful probe for determining the fine structure of such biopolymers.

In certain cases, noncarbohydrate substituents on polysaccharide antigens may constitute an immunodominant group, but, in the present instance, the *O*-acetyl

group does not appear to fulfil this function, as the bacteriophage appears equally able to depolymerize the native and the deacetylated K54 polysaccharide.

The results presented here make a minor correction to the structure of the capsular polysaccharide from *Klebsiella* serotype K54 and also demonstrate that the phage-borne enzyme has a β-D-glucosidase activity. This is consistent with the results of Stirm and Rieger-Hug[17], from whom the bacteriophage was obtained, but at variance with the results reported by Sutherland[1].

A reinvestigation of some of the oligosaccharides obtained previously, and kindly provided by Dr. Sutherland, show them to be identical with the oligomers isolated in the present study.

EXPERIMENTAL

General methods. — Paper chromatography was conducted by using Whatman No. 1 paper and the following solvent systems (all v/v) (*1*) 2:1:1 1-butanol–ethanol–water, (*2*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, and (*3*) 4:1:5 (upper phase) 1-butanol–ethanol–water. Preparative paper-chromatography was performed with Whatman No. 3 MM paper and freshly prepared solvent *2*. Analytical g.l.c. separations were performed in stainless-steel columns (1.8 m × 3 mm) with a carrier-gas flow-rate of 20 mL/min. Columns used were (*A*) 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh) isothermally at 180° for 4 min, and then programmed at 2°/min to 200°; and (*B*) 3% of OV-17 on Gas-Chrom Q (100–120 mesh) isothermally at 160° for 4 min, and then programmed at 4°/min to 230°.

Methylation of poly- and oligo-saccharides was achieved by the modified, Hakomori procedure described earlier[8,9]. Methylated derivatives were carboxyl-reduced with lithium aluminum hydride in anhydrous oxolane at room temperature. Sugars present in the hydrolyzates of methylated and methylated, carboxyl-reduced samples were examined by paper chromatography in solvent *3*, and quantitatively analyzed by g.l.c. of their partially methylated alditol acetates in column *A*.

Other general method employed in this study have been described[9].

A. Analysis of K54 polysaccharide

Methylation analyses. — Samples (20–30 mg) of the K54 polysaccharide in the acid form were methylated according to the procedures described previously[9]. A portion of the methylated derivative obtained in each case was carboxyl-reduced, hydrolyzed with 2M trifluoroacetic acid, and the hydrolyzate analyzed by paper chromatography (solvent *3*) and by g.l.c., to give the results shown in Table II, columns II–IV.

A sample of the K54 polysaccharide was de-esterified with 0.1M KOH on a steam bath for 18 h, dialyzed, and the dialyzate freeze-dried. P.m.r. spectroscopy of the polysaccharide indicated the complete removal of the *O*-acyl groups. The de-acylated polysaccharide was methylated (10 sec, in base), the product was hydrolyzed, and the sugars present in the hydrolyzate were analyzed by paper chromatography

and by g.l.c. (column *A*), to give the proportions of sugars shown in Table II. A further portion of the methylated derivative was carboxyl-reduced, and the product analyzed similarly (see Table II). No evidence was obtained either from i.r. spectroscopy or from g.l.c. to suggest undermethylation in these methylated derivatives.

Periodate oxidation. — The native and the deacetylated K54 polysaccharide were treated with 0.1M $NaIO_4$ in the presence of $NaClO_4$ (0.4M) for 72 h at 4° in the dark. At the end of this period, ethylene glycol was added, the solutions were stirred at room temperature for 1 h, dialyzed overnight against running tap-water, and the products reduced with sodium borohydride for 3 h. Acidification with Amberlite IR-120 (H^+) resin, filtration, removal of the borate by dialysis, and freeze-drying, yielded the polyalcohol. A portion of each polyol was hydrolyzed with 2M trifluoroacetic acid. Paper chromatography (solvents *1* and *2*) of both hydrolyzates showed the presence of glycolaldehyde, glycerol, erythritol, fucose, and glucose, which were converted into a mixture of their derived alditol acetates and this analyzed by g.l.c. in column *B* (see Table III). A second portion of each polyalcohol was hydrolyzed with M trifluoroacetic acid for 18 h at room temperature, and the hydrolyzate examined by paper chromatography (solvent *1*), which showed the presence of glycolaldehyde, glycerol, erythritol, and a slower-moving component which was isolated by preparative paper-chromatography and then methylated. Analysis of each of the methylated derivatives by paper chromatography and by g.l.c. (column *A*) showed 2,4,6-tri-*O*-methylglucose and 2,4-di-*O*-methylfucose in equimolar proportions.

B. Bacteriophage-induced depolymerization

Isolation and characterization of oligosaccharides. — Samples of the native and the deacetylated K54 polysaccharides were depolymerized with the host bacteriophage ϕ54 according to the procedures recently described[14]. Oligosaccharides were separated into pure components by preparative paper-chromatography, and these analyzed by standard techniques, to give the results shown in Table IV. Methylation analyses of oligosaccharides **1** and **4** obtained by the methods referred to earlier are shown in Table V.

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