STRUCTURAL STUDIES OF THE *Klebsiella* TYPE 9 CAPSULAR POLYSACCHARIDE

BENGT LINDBERG, JÖRGEN LÖNNGREN, JAMES L. THOMPSON*,
Institutionen för organisk kemi, Stockholms universitet, S-113 27 Stockholm (Sweden)

AND WOLFGANG NIMMICH
Institut för Medizinische Mikrobiologie und Epidemiologie der Universität Rostock, 25 Rostock (D.D.R.)

(Received March 9th, 1972; accepted for publication, March 17th, 1972)

ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* type 9 has been investigated. Methylation analysis and characterization of oligosaccharides obtained on mild acid hydrolysis were the principal methods used. The polysaccharide is composed of pentasaccharide repeating units, containing D-glucuronic acid, D-galactose, and L-rhamnose in the ratios 1:1:3, and a structure for these units is proposed.

INTRODUCTION

The chemical compositions of type-specific capsular polysaccharides (K-antigens) from *Klebsiella* types 1–80 have been investigated by Nimmich[1,2]. For the K2[3], K8[4], K21[5], and K54[6,7] antigens, full or partial structures have been determined. The structure of another *Klebsiella* K-antigen (from *Aerobacter aerogenes* DD-45) has also been determined[8]. All these K-antigens are acidic polysaccharides, composed of oligosaccharide repeating units. The presence of repeating units has also been confirmed in biosynthetic studies. In the present communication, structural studies of the K-antigen from *Klebsiella* type 9 are reported.

RESULTS AND DISCUSSION

The polysaccharide was isolated as previously described[1], and some impurities of low molecular weight were removed by dialysis. It had $[\alpha]_{578}$ −17°. It did not show any absorption in the 1735 cm^{-1} region in the i.r., demonstrating the absence of *O*-acetyl or other *O*-acyl groups. On acid hydrolysis, the polysaccharide yielded glucuronic acid, galactose, and rhamnose[1]. Glucose, galactose, and rhamnose were obtained from a hydrolysate of the polysaccharide *via* reduction of the trimethylsilyl

*On leave from the Collège militaire royal de Saint-Jean, Québec, Canada.

ethers with lithium aluminium deuteride. The relative proportions 1.0:1.0:2.9 and the presence of two deuterium atoms at C-6 in the glucose were demonstrated by g.l.c.[9]–m.s.[10] of the alditol acetates derived from the sugars. D-Galactose and L-rhamnose were isolated from a hydrolysate, and their configurations proved by their optical rotations. The D-configuration of the glucuronic acid was demonstrated by the action of β-glucuronidase on the aldobiouronic acid (oligosaccharide A_1).

The polysaccharide was methylated by the Hakomori procedure[11], and part of the product was reduced with lithium aluminium deuteride. The two samples were hydrolysed and the sugars in the hydrolysates were analysed, as their alditol acetates, by g.l.c.–m.s.[12,13]. The results are summarized in Table I. The components were readily identified from their retention times and mass spectra. 2,3,4-Tri-*O*-methyl-D-glucose, only obtained from the carboxyl-reduced products, was dideuterated at C-6 and was thus derived from terminal D-glucopyranosyluronic acid residues.

TABLE I

METHYL ETHERS FROM THE HYDROLYSATE OF THE METHYLATED (*A*) AND METHYLATED, CARBOXYL-REDUCED POLYSACCHARIDE (*B*)

Sugars[a]	T[b]	*Mole %*	
		A	B
3,4-Rha	0.85	22.6	22.4
2,4-Rha	0.93	23.4	17.5
2-Rha	1.37	29.0	24.1
2,4,6-Gal	2.03	25.0	18.6
2,3,4-G	2.22		17.5

[a]3,4-Rha = 3,4-di-*O*-methyl-L-rhamnose, etc. [b]Retention time of the corresponding alditol acetate, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 column at 170°.

There is good agreement between the sugar analysis and the methylation analysis. The results strongly suggest that the polysaccharide contains a pentasaccharide repeating unit with one D-glucuronic acid residue, one D-galactose residue, and three L-rhamnose residues. The analysis also gives information on the positions through which these residues are linked.

In order to obtain information about the arrangement of the sugar residues, the polysaccharide was subjected to partial hydrolysis with acid, and several oligosaccharides were isolated. Three acidic oligosaccharides (A_1, A_2, and A_3) and one neutral oligosaccharide (*N*) were examined more closely. Each gave a single spot on paper chromatography, as did the acidic oligosaccharides on paper electrophoresis in acetate buffer of pH 4. The permethylated alditols, prepared by reduction with sodium borodeuteride and Hakomori methylation[11] (etherification and esterification), were homogeneous on g.l.c. Some of the properties of the oligosaccharides are summarized in Table II. The values for the optical rotations of the oligosaccharides are not very accurate because of the small amounts available.

TABLE II

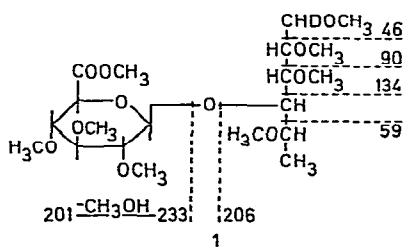
PHYSICAL PROPERTIES OF THE OLIGOSACCHARIDES

Oligosaccharide	*Yield (mg)*	$[\alpha]_{578}^{20}$ *(degrees)*	*Paper-chromatographic mobility*[a]	T_{MEL}[b]	*Methyl ethers from the hydrolysates of the methylated oligosaccharide alditols*[d,e]
A_1	14.0	−34	0.64	0.54	2,3,4-G[f] 1,2,3,5-Rha[g]
A_2	2.8	−21	0.57	1.14	2,3,4-G[f] 2,3-Rha 1,2,4,5-Rha[g]
A_3	5.1	+22	0.36	6.30[c]	2,3,4,6-Gal 2,3,4-G[f] 1,2,5-Rha[g]
N	7.8	−21	0.43	2.50	3,4-Rha 2,3,4-Rha 1,2,4,5,6-Gal[g]

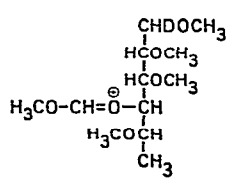
[a]Relative to L-rhamnose, solvent system *A*. [b]Retention time of the permethylated alditol on the XE-60 column relative to permethylated melibiitol at 200°. [c]At 220°. [d]Identified by g.l.c.–m.s. of the corresponding alditol acetates on an ECNSS-M column. The acidic oligosaccharides were reduced by lithium aluminium deuteride before hydrolysis. [e]2,3,4-G = 2,3,4-tri-*O*-methyl-D-glucose, etc. [f]Deuterium labelling in the 6-position. [g]Deuterium labelling in the 1-position.

Hydrolysis of the methylated oligosaccharide alditols (after carboxyl reduction of the acidic ones) and analysis of the products in the hydrolysates, as the alditol acetates, gave information on their structures. Complementary evidence was obtained by g.l.c.–m.s. of methylated oligosaccharide alditols. Systematic studies by Kochetkov[14], Bauer[15,16], and Kärkkäinen[17,18] and their co-workers facilitated the interpretation of the latter spectra.

From the acidic oligosaccharide A_1, 2,3,4-tri-*O*-methyl-D-glucose, dideuterated at C-6, and 1,2,3,5-tetra-*O*-methyl-L-rhamnitol, monodeuterated at C-1, were obtained. This, in conjunction with the low optical rotation, demonstrates that A_1 is 4-*O*-(β-D-glucopyranosyluronic acid)-L-rhamnose[19]. The optical rotation indicates that the configurations of the two sugar residues are those shown above, and this was corroborated by the action of β-glucuronidase on the oligosaccharide. M.s. data for the permethylated alditol methyl ester were in good agreement with this structure.



1

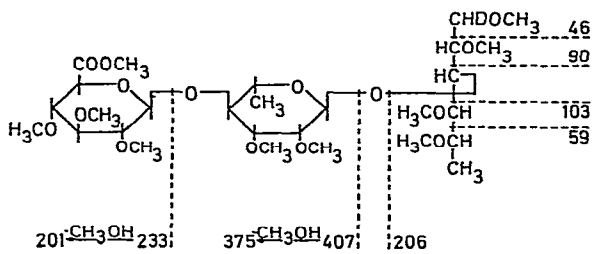


m/e 266

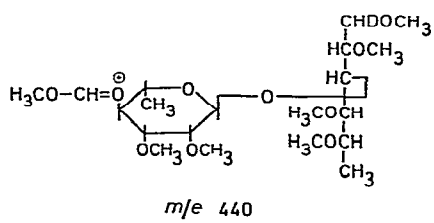
2

The origins of important fragments are indicated in **1**. In addition to these, a rearranged fragment **2**, *m/e* 266, containing the intact alditol part, was observed.

From the acidic fragment A_2, 2,3,4-tri-*O*-methyl-D-glucose, dideuterated at C-6, 2,3-di-*O*-methyl-L-rhamnose (*T*-value of the alditol acetate, 0.98), and 1,2,4,5-tetra-*O*-methyl-L-rhamnitol, monodeuterated at C-1, were obtained. This, in conjunction with the low optical rotation, demonstrates that A_2 has the structure β-D-GA*p*-(1→4)-α-L-Rha*p*-(1→3)-L-Rha*p*. The mass spectrum of the permethylated alditol methyl ester was in good agreement with this structure. In addition to the fragments produced by fission of linkages and further eliminations (as indicated in 3), **4** (*m/e* 440) and a fragment analogous to **2** (*m/e* 266) were observed. The sequence of the three sugar residues and the (1→3)-linkage to the terminal L-rhamnitol part are evident from the m.s. The absence of a strong fragment *m/e* 375 (407 − 32), which would have indicated that the central L-rhamnose was linked in the 2-position, and the presence of a fragment *m/e* 266, which would similarly have been absent if the central L-rhamnose were linked in the 3-position, strongly suggest that it is linked in the 4-position[18]. This is already demonstrated by other evidence.

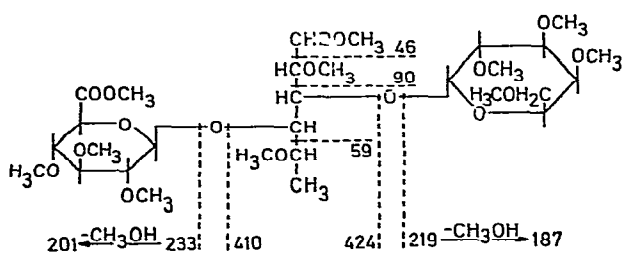


3

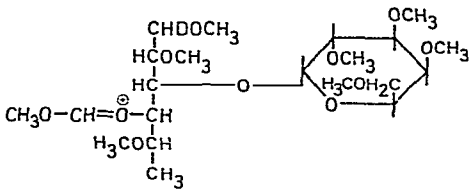


4

From the acidic oligosaccharide A_3, 2,3,4-tri-*O*-methyl-D-glucose, dideuterated at C-6, 2,3,4,6-tetra-*O*-methyl-D-galactose and 1,2,5-tri-*O*-methyl-L-rhamnitol, monodeuterated at C-1 (*T*-value of the acetate, 0.35), were obtained. Oligosaccharide A_3 is consequently a branched trisaccharide, in which terminal D-galactose and terminal D-glucuronic acid are linked to C-3 and C-4 in L-rhamnose. It is known from other evidence that the D-glucuronic acid residue is β-linked, but it is difficult to decide from the optical rotation of A_3 whether the D-galactose residue is α- or β-linked. The mass spectrum of the permethylated oligosaccharide alditol methyl ester is in agree-

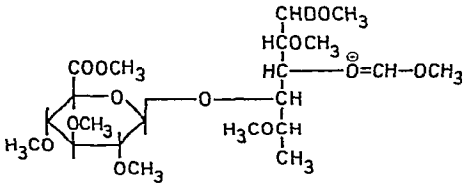


5.



m/e 470

6

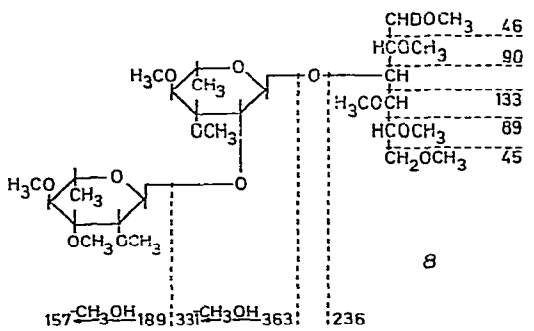


m/e 484

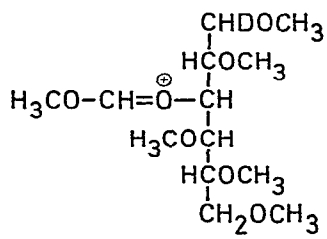
7

ment with this structure. In addition to fragments found by fission of the linkages and further eliminations, as indicated in **5**, the rearranged fragments **6** and **7**, both containing the alditol part and one of the terminal sugar residues, are those expected for the proposed structure.

From the neutral oligosaccharide *N*, 2,3,4-tri-*O*-methyl-L-rhamnose, 3,4-di-*O*-methyl-L-rhamnose, and 1,2,4,5,6-penta-*O*-methyl-D-galactitol, monodeuterated at C-1 (*T*-value of the acetate, 0.42), were obtained. From this evidence, in conjunction with the low value for its optical rotation, the structure of *N* is α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-D-Gal*p*. The mass spectrum of the permethylated alditol is in agreement with this structure. In addition to the fragments obtained by fission of linkages and further eliminations, as indicated in **8**, the rearranged fragments **9** (*m/e* 296) and **10** (*m/e* 470) were observed. The sequence of the three sugar residues and the (1→3)-linkage to the D-galactitol residue are immediately evident. The strong fragment of *m/e* 331 (363−32) suggests that the central L-rhamnose residue is linked to the 2-position[18], as demonstrated by other evidence.

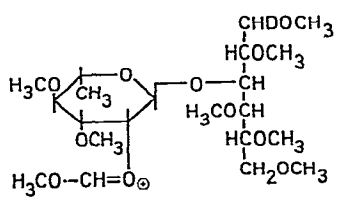


8



m/e 296

9

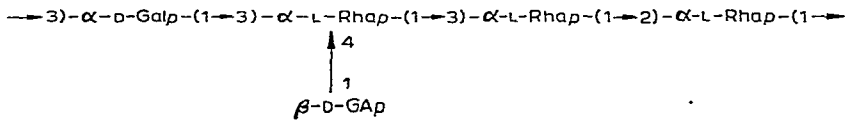


m/e 470

10

From the optical rotations of the oligosaccharides, it was evident, as discussed above, that the D-glucuronic acid residue is β-linked and that the three L-rhamnose residues are α-linked. However, the configuration of the D-galactose residue could not be so easily decided. In general, oligosaccharide alditol acetates containing β-links are oxidized by chromium trioxide in acetic anhydride much more readily than those containing α-links[20,21]. A partially purified, higher oligosaccharide, obtained on partial hydrolysis with acid, was therefore reduced with borohydride, acetylated, and treated with chromium trioxide in acetic anhydride. *myo*-Inositol hexa-acetate was added as an internal standard. Under the conditions used, most β-D-galactose residues would have been oxidized. Sugar analysis revealed that only a small part of the D-galactopyranose residues had been affected. As the D-galactitol obtained on hydrolysis and borodeuteride reduction of the product contained deuterium at C-1, the D-galactose residues did not occupy reducing, terminal positions in the oligosaccharide. The fact that they resisted oxidation therefore demonstrates that they are α-linked.

From the combined evidence presented above, the structure **11** for the pentasaccharide repeating unit of the K-antigen from *Klebsiella* type 9 can be proposed. This structure could be written in four different cyclic permutations. It cannot be decided which of these is the "biological" repeating unit, as no methylated sugar derived from the non-reduced terminal was observed in the methylation analysis.



11

EXPERIMENTAL

General methods. — Concentrations were carried out under diminished pressure at bath temperatures not exceeding 40°. For g.l.c., Perkin–Elmer 900 or 990 instruments, fitted with flame-ionisation detectors, were used. Separations were performed on glass-columns (180 × 0.15 cm) containing (*a*) 3% OV-225 on Gas Chrom Q (100/120 mesh) at 170° (partially methylated alditol acetates), (*b*) 3% ECNSS-M on the same support at 190° (alditol acetates) or at 170° (partially methylated alditol acetates), and (*c*) 5% XE-60 on Chromosorb W (80/100 mesh) at 200° or 220° (permethylated oligosaccharide alditol derivatives). For quantitative evaluation of the g.l.c., a Hewlett Packard 3370B integrator was used. For mass-spectrometry a Perkin–Elmer 270 g.l.c.–m.s. instrument fitted with the above-mentioned columns was used. Mass spectra were recorded at an ionisation potential of 70 eV, an ionisation current of 80 μamp, and an ion-source temperature of 80°. Paper chromatography was performed on Whatman No. 1 paper in the solvent system (*A*) ethyl acetate–acetic acid–water (3:1:1). For preparative purposes, FILTRAK FN-4 paper in the solvent

system (*B*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4) or Whatman No. 1 paper in solvent system *A* was used. Electrophoresis was performed on Whatman No. 1 paper in acetate buffer of pH 4.0 at 40 volts/cm for 70 min. The compounds were detected with 3% *p*-anisidine hydrochloride in ethanol at 120°. Optical rotations were recorded using a 10-cm micro-cell with a Perkin–Elmer 141 instrument, and i.r. spectra were recorded in a Perkin–Elmer 257 instrument.

Isolation of the polysaccharide. — The isolation from strain *Klebsiella* 56 (K-type 9) was performed as previously described[1]. Before analyses were carried out, the material was dialysed against distilled water and lyophilized. The polysaccharide showed $[\alpha]_{578}^{20}$ −17° (*c* 0.2, water). In the i.r. spectrum (KBr), no significant absorptions around 1735 cm^{-1} (*O*-acyl region) were observed.

Sugar analysis. — The polysaccharide (10 mg) in 0.25M sulphuric acid (3 ml) was kept at 100° for 12 h. The hydrolysate was neutralised with barium carbonate, filtered, and concentrated to dryness. The product was dried *in vacuo* over phosphorus pentaoxide, dissolved in dry pyridine (1.5 ml), and trimethylsilylated using hexamethyldisilazane (0.3 ml) and chlorotrimethylsilane (0.15 ml) as silylation agents. After work-up, the TMS derivatives were treated with lithium aluminium deuteride (50 mg) in boiling ethyl ether (10 ml) for 6 h. After processing, the product was hydrolysed with 0.25M sulphuric acid for 12 h at 100°, the acid was neutralised with barium carbonate, and the sugars were converted into alditol acetates as previously described[22]. The mixture was then analysed by g.l.c.[9]–m.s.[10].

A larger sample of polysaccharide (50 mg) was hydrolysed as above, neutralised with barium carbonate, and concentrated to dryness. Rhamnose and galactose were isolated by preparative, paper chromatography (Whatman No. 1, solvent system *A*). The syrupy samples showed $[\alpha]_{578}^{20}$ +6° (*c* 1.3, water) and $[\alpha]_{578}^{20}$ +54° (*c* 0.5, water), respectively.

Methylation analyses. — The polysaccharide (10 mg) in methyl sulphoxide (2 ml) was treated with 2M methylsulphinyl sodium (2 ml) under nitrogen. The resulting solution was agitated in an ultrasonic bath for 1 h and then kept for 2 h at room temperature. Methyl iodide (4 ml) was then added dropwise with external cooling. The turbid solution was agitated in an ultrasonic bath for 1 h, when a clear solution was obtained. The mixture was then dialysed against running tap-water overnight and concentrated to dryness. Half of the material was treated with 90% formic acid (2 ml) at 100° for 1 h, the solution was concentrated to dryness, and the residue was dissolved in 0.13M sulphuric acid and kept for 12 h at 100°. The hydrolysate was neutralised with barium carbonate, and the sugars were converted into alditol acetates[22] and analysed by g.l.c.–m.s.[12,13].

The other half of the methylated polysaccharide was dissolved in dry ethyl ether (35 ml), lithium aluminium deuteride (100 mg) was added, and the reaction mixture was refluxed for 2 h. After processing, the product was hydrolysed as above, and the methylated sugars were converted into alditol acetates[22] and analysed by g.l.c.–m.s.[12,13].

Isolation of oligosaccharides. — The polysaccharide (300 mg) was hydrolysed in

0.13M sulphuric acid (30 ml) at 100° for 1 h. The hydrolysate was neutralised with barium carbonate. The solution was added to the top of a column (15 × 0.9 cm) of Dowex 1-x8 (OAc) resin which was eluted first with water, yielding neutral oligosaccharides, and then with a gradient (0–6%) of acetic acid, yielding a series of acidic oligosaccharides. The oligosaccharides were further purified by preparative, paper chromatography (FILTRAK FN-4, solvent system *B*).

Characterisation of oligosaccharides. — All the characterised oligosaccharides gave single spots on paper chromatography (the mobilities are given in Table II). On paper electrophoresis in acetate buffer, the acidic oligosaccharides appeared to be homogeneous. The oligosaccharides (2 mg) were transformed into alditols by reduction in water (5 ml) with sodium borodeuteride (20 mg). After treatment of the reaction mixtures with Dowex-50 (H^+) resin and concentration, boric acid was removed by co-distillation with methanol. The oligosaccharide alditols were then methylated by Hakomori's procedure as described above, except that the methylated products were isolated by partition between chloroform and water. The methylated oligosaccharide alditols were analysed by g.l.c.–m.s.[17,18], using an XE-60 column. The m.s. of the components showed, *inter alia*, the following peaks (relative intensities in brackets): A_1: 46(28), 59(68), 88(40), 90(10), 101(70), 134(15), 169(13), 201(100), 206(15), 233(18), 266(4); A_2: 46(8), 59(27), 88(86), 90(10), 101(58), 103(10), 169(6), 201(100), 206(7), 233(10), 266(20), 407(6), 440(7); A_3: 46(6), 59(22), 88(100), 90(10), 101(80), 169(6), 187(56), 201(94), 219(21), 233(10), 410(0.4), 424(1.4), 470(0.1), 484(1.2); *N*: 45(60), 46(11), 88(98), 89(32), 90(21), 101(100), 133(10), 157(56), 189(94), 236(38), 296(13), 331(7), 363(10), 378(11), 410(1), 470(4).

In order to transform the methylated acidic oligosaccharides into alditol acetates, the preparations were dissolved in dry ethyl ether (25 ml), and lithium aluminium deuteride (100 mg) was added. The solutions were refluxed for 3 h. After processing, the products were hydrolysed with 0.13M sulphuric acid for 12 h at 100°, and the methylated sugars were converted into alditol acetates and analysed by g.l.c.–m.s.[12]. The permethylated alditol from the neutral oligosaccharide *N* was hydrolysed directly, and the products were transformed into alditol acetates, using the same procedures described above, and analysed by g.l.c.–m.s.[12]. *T*-values for the tetra-*O*-methyl-L-rhamnitol derivatives from oligosaccharides A_1 and A_2 were too low to be determined accurately.

Configuration of glucuronic acid. — The aldobiuronic acid (1 mg) was incubated (37°) overnight with β-glucuronidase (Sigma Chemical Company) in 4mM phosphate buffer pH 6.8 (0.5 ml). Buffer ions were removed by successive treatment with silver acetate and Dowex-50 (H^+) resin. Paper chromatography (solvent system *A*) showed that the oligosaccharide had been hydrolysed to glucuronic acid and rhamnose.

Determination of anomeric configurations by chromium trioxide oxidation. — A partially purified, higher oligosaccharide (2 mg) was reduced with sodium borohydride and acetylated with acetic anhydride in pyridine. The alditol acetate was dissolved in acetic anhydride (0.2 ml), and *myo*-inositol hexa-acetate (0.4 mg) was added as an internal standard. One half of the sample was then subjected to sugar analysis as

described below, while to the remainder of the sample acetic anhydride (0.1 ml) containing chromium trioxide (100 mg/ml) was added[21]. After 1 h at room temperature, this mixture was diluted with water (5 ml) and extracted with chloroform (3 × 5 ml), and the extract was washed with water (3 × 5 ml). The extract was then concentrated to dryness, products were hydrolysed overnight with 0.25M sulphuric acid at 100°, neutralised (barium carbonate), and reduced with sodium borodeuteride, in order to distinguish between chain and terminal alditol residues. The alditol mixture was then acetylated with acetic anhydride in pyridine and examined by g.l.c.[9]–m.s.[10]. The proportions of rhamnitol penta-acetate, galactitol hexa-acetate, and *myo*-inositol hexa-acetate were essentially the same as in the standard sample.

ACKNOWLEDGMENTS

The skilled technical assistance of Mrs. Jana Cederstrand is acknowledged. This work was supported by grants from the Swedish Natural Science Research Council, from the Swedish Medical Research Council (B72-40X-2522-04), from Harald Jeanssons Stiftelse, and from Stiftelsen Sigurd och Elsa Goljes Minne.

REFERENCES

1 W. NIMMICH, *Z. Med. Microbiol. Immunol.*, 154 (1968) 117.
2 W. NIMMICH, *Acta Biol. Med. Ger.*, 26 (1971) 397.
3 L. C. GAHAN. P. A. SANDFORD, AND H. E. CONRAD, *Biochemistry*, 6 (1967) 2755.
4 I. W. SUTHERLAND, *Biochemistry*, 9 (1970) 2180.
5 G. G. S. DUTTON AND Y. M. CHOY, *Carbohyd. Res.*, 21 (1972) 169.
6 P. A. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508.
7 H. E. CONRAD, J. R. BAMBURG, J. D. EPLEY, AND T. J. KINDT, *Biochemistry*, 5 (1966) 2808.
8 E. C. YUREWICZ, M. A. GHALAMDOR, AND E. C. HEATH, *J. Biol. Chem.*, 246 (1971) 5596.
9 J. S. SAWARDEKER, J. H. SLONEKER, AND A. R. JEANES, *Anal. Chem.*, 37 (1965) 1602.
10 O. S. CHIZHOV, L. S. GOLOVKINA, AND N. S. WOLFSON, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1966) 1915.
11 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205.
12 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem.*, 82 (1970) 643.
13 J. LÖNNGREN AND Å. PILOTTI, *Acta Chem. Scand.*, 25 (1971) 1144.
14 N. K. KOCHETKOV AND O. S. CHIZHOV, *Advan. Carbohyd. Chem.*, 21 (1966) 39.
15 V. KOVÁČIK, Š. BAUER, J. ROSIK, AND P. KOVÁČ, *Carbohyd. Res.*, 8 (1968) 282.
16 V. KOVÁČIK, Š. BAUER, AND J. ROSIK, *Carbohyd. Res.*, 8 (1968) 291.
17 J. KÄRKKÄINEN, *Carbohyd. Res.*, 14 (1970) 27.
18 J. KÄRKKÄINEN, *Carbohyd. Res.*, 17 (1971) 11.
19 P. J. MCKINNELL AND E. PERCIVAL, *J. Chem. Soc.*, (1962) 2082.
20 J. HOFFMAN, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 26 (1972) 661.
21 J. HOFFMAN, B. LINDBERG, AND S. SVENSSON, to be published.
22 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801.