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STRUCTURAL STUDIES OF SOME BACTERIAL POLYSACCHARIDES

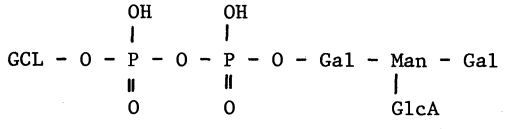
Bengt Lindberg

Department of Organic Chemistry, University of Stockholm,
S-104 05 Stockholm, Sweden

INTRODUCTION

Organic chemists have, for a considerable time, used biosynthetic principles as an aid in structural studies of naturally occurring substances. Among the most important examples have been the "isoprene rule" and the "acetate hypothesis". When chemical studies have not discriminated between alternative structures, some of these could often be excluded as they were inconsistent with the biosynthetic principles. For similar reasons, it has also been predicted and later proved that proposed structures for natural products were erroneous. There is no reason why use should not be made of similar principles in polysaccharide chemistry, especially in structural studies of bacterial polysaccharides.

Biosynthetic studies have revealed that extracellular polysaccharides and the O-antigenic side chains of cell wall lipopolysaccharides are composed of oligosaccharide repeating units (Ref. 1). In the biosynthesis of these polymers, the monosaccharides are transferred sequentially from their nucleotide precursors to the phosphoric monoester of an isoprenoid, C_{55} alcohol, glycosyl carrier lipid (GCL). The first sugar is transferred as a glycosyl-1-phosphate and the others as simple glycosyl moieties. The complete repeating unit is then transferred from the intermediate (for example 1) to the growing polymer chain.



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Although these biosynthetic processes have only been studied in detail for a limited number of polysaccharides, the results are most probably of general validity. This hypothesis that bacterial polysaccharides are composed of oligosaccharide repeating units is useful in that it greatly facilitates their structural analysis. It should be noted, however, that some groups, such as O-acetyl and terminal α-D-glucopyranosyl groups in O-antigens, appear to be added at a later stage of the biosynthetic process and also do not have to occur in stoichiometric amounts.

The synthesis of a polysaccharide composed of repeating units requires a multitude of highly specific enzymes, the number of which increases with the complexity of the repeating unit. Most repeating units, however, seem to be rather small, di- to hexasaccharides having been demonstrated for several polysaccharides. It therefore seems to be a good working hypothesis that a repeating unit is fairly simple. Further justification for this comes from the reinvestigation of bacterial polysaccharides for which large repeating units have been proposed. These have consistently led to revised structures of a more simple nature.

Some extracellular polymers from bacteria are composed of oligosaccharide repeating units, linked to each other by means of phosphoric diester linkages. The biosynthesis of these polymers, and the related teichoic acids, seems to be analogous to that of the polysaccharides discussed above, except that the oligosaccharide is transferred to the polymer together with a phosphate group (Ref. 2). In addition to sugar residues, glycerol phosphate or ribitol phosphate may also be part of these polymers. These units are transferred from CDP-glycerol and CDP-ribitol, in which the phosphate is linked to O-1 of D-glycerol and to O-5 of D--ribitol, respectively. Consequently these polymers should contain phosphate linked to one of these positions or to O-1 of a glycosyl residue. The latter should have the same anomeric configuration as in the corresponding "sugar nucleotide". These biosynthetic considerations are useful as complementary evidence in structural studies.

In the following I shall discuss structural studies of some bacterial polysaccharides which will illustrate these principles and also exemplify some new methods in structural polysaccharide chemistry.

SHIGELLA FLEXNERI POLYSACCHARIDES

Shigella flexneri is one of the organisms causing dysentery, a disease which is a serious problem for many countries and a menace to others. The immunological properties of the organism are, to a great extent, determined by the O-specific side chains in the cell wall lipopolysaccharide (LPS) and structural studies of this is therefore a matter of some importance. Although there are different serotypes of S. flexneri, the O-specific side chains in these seem to have the same basic structure. Variations are provided by adding terminal α-D-glucopyranosyl groups or O-acetyls to different positions. Various repeating units (2, 3 (Ref. 3), 4 (Ref. 4) and 5 (Ref. 5) have been suggested to make up this basic structure.

→6)-D-GlcNAcp-(1→2)-L-Rhap-(1→4)-L-Rhap-(1→

2

→4)-D-GlcNAcp-(1→3)-L-Rhap-(1→4)-L-Rhap-(1→

3

→6)-D-GlcNAcp-(1→2)-L-Rhap-(1→3)-L-Rhap-(1→

4

→3)-D-GlcNAcp-(1→2)-L-Rhap-(1→2/3)-L-Rhap-(1→3/2)-L-Rhap-(1→

5

No information on the anomeric nature of the linkages is given in any of these. We are now reinvestigating these LPS (Ref. 6), and have started with that from S. flexneri variant Y, as this should not contain α-D-glucopyranosyl groups.

Sugar analysis of a polysaccharide containing 2-acetamido-2-deoxy-D-glucose residues is not very accurate but the 100 MHz n.m.r. spectrum (Fig. 1) of the polysaccharide obtained from the LPS on mild acid hydrolysis was informative. The area of the signal given by methyl

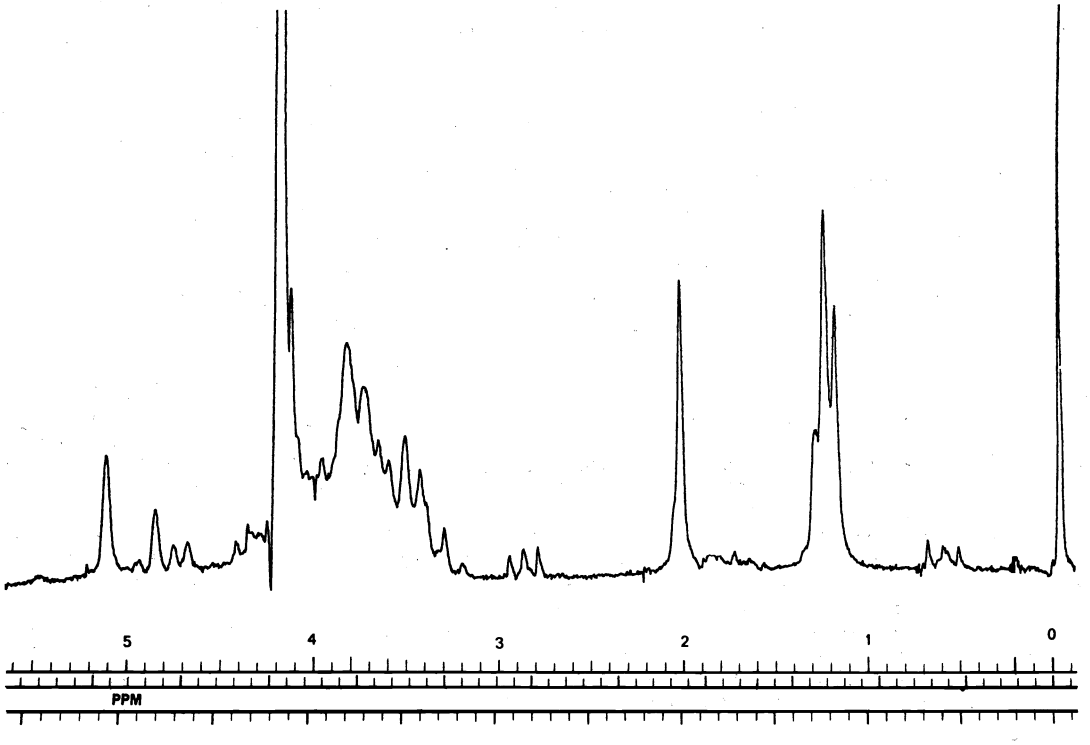


Fig. 1. N.m.r. spectrum of S. flexneri O-antigen.

protons of L-rhamnose residues was three times larger than that given by the methyl protons of N-acetyl groups, thus confirming our proposal (Ref. 5) that the repeating unit is a tetrasaccharide with one 2-acetamido-2-deoxy-D-glucose and three L-rhamnose residues. There are four protons in the anomeric region and one of these, from its shift (δ = 4.7) and coupling constant (J_{12} = 7 Hz), is given by a 2-acetamido-2-deoxy-β-D-glucopyranosyl group. A two-proton signal at δ 5.1 probably derives from α-linked L-rhamnopyranose residues, but it is difficult to tell if the signal at δ 4.8 derives from a β- or α-linked L-rhamnopyranose residue.

Methylation analysis (Table 1) showed that two of the L-rhamnopyranose residues were linked through O-2 and the third through O-3, whilst the 2-acetamido-2-deoxy-D-glucose was substituted at O-3. In a separate experiment the polysaccharide was fully N-deacetylated before

TABLE 1. Methylation analysis of the S. flexneri O-antigen

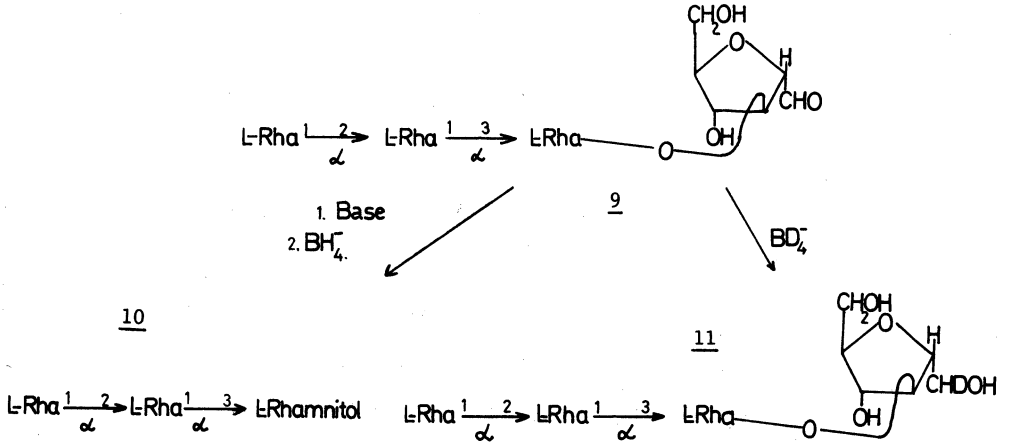
Sugar	A[1)]	B[2)]
3,4-Di-OMe-L-Rha	2	1
2,4-Di-OMe-L-Rha	1	1
2,4-Di-OMe-D-GlcNAc	+	-

1) A, original polysaccharide
2) B, N-deacetylated polysaccharide

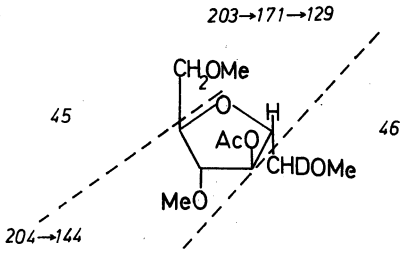
the methylation analysis. In the methylation analysis of this product, the glucosaminidic linkage was resistant to acid hydrolysis and the glucosamine and the sugar to which it was linked were not observed. The analysis consequently demonstrates the structural elements 6, 7 and 8.

→3)-D-GlcNAcp-(1→2)-L-Rhap-(1→ (6) →2)-L-Rhap-(1→ (7) →3)-L-Rhap-(1→ (8)

A new technique was used for N-deacetylation of the polysaccharide; treatment with sodium hydroxide and sodium thiophenolate in aqueous dimethyl sulfoxide (Ref. 7). Treatment of the fully N-deacetylated polysaccharide with nitrous acid yielded the tetrasaccharide 9. Part of 9 was treated with base, when the trisaccharide residue in the β-position to the aldehyde



group was eliminated. This trisaccharide was reduced with borohydride and the product (10) subjected to methylation analysis. The identification of 2,3,4-tri-O-methyl-L-rhamnose, 3,4-di-O-methyl-L-rhamnose and 1,2,4,5-tetra-O-methyl-L-rhamnitol demonstrated that the central L-rhamnose residue was linked through O-2 and was glycosidically linked to O-3 of the following L-rhamnose. Another part of 9 was reduced with borodeuteride to 11 and subjected to methylation analysis. In addition to the expected L-rhamnose derivatives, a tri-O-methyl derivative of 2,5-anhydro-D-mannitol was obtained. That this was the 1,4,6-tri-O-methyl derivative was demonstrated by m.s. of its acetate (12).



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The primary fragment m/e 203, formed by fission between C-1 and C-2, lost first methanol, by β-elimination, and then ketene. The primary fragment m/e 204, formed by fission between C-5 and C-6, however, lost acetic acid, also by β-elimination. The observation that glucosamine is linked through O-3 was thereby confirmed.

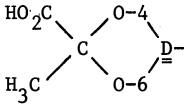
As discussed above, n.m.r. results indicated that at least two of the L-rhamnose residues are α-linked. The difference in chemical shifts between α- and β-anomers is not large. The magnitudes of the shifts also varied considerably for the polysaccharide, the N-deacetylated polysaccharide and the oligosaccharides 10 and 11. The values for the optical rotations of 10 and 11, $[\alpha]_D$ - 44° and $[\alpha]_D$ - 30°, respectively, indicate, however, that all three L--rhamnose residues are α-linked. From the combined evidence, the structure 13 is proposed for the basic repeating unit in the S. flexneri O-antigen. The structures of the antigens from the other serogroups are now under investigation by similar methods.

→3)-β-D-GlcNAcp-(1→2)-α-L-Rhap-(1→2)-α-L-Rhap-(1→3)-α-L-Rhap-(1→

13

THE CAPSULAR POLYSACCHARIDE FROM RHIZOBIUM MELILOTI

Some years ago we investigated the capsular polysaccharide from Rhizobium meliloti (Ref. 8), a nitrogen fixating organism. The polysaccharide, which showed $[\alpha]_{578}$ - 4°, contained D--glucose, D-galactose, pyruvic acid residues and O-acetyl in the approximate proportions 7:1:1:1. Methylation analysis revealed the following structural elements:



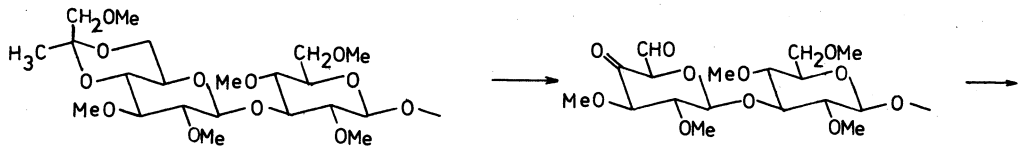
→4)-D-Glcp-(1→ → 3)-D-Galp-(1→

→6)-D-Glcp-(1→ →4)-D-Glcp-(1→ →3)-D-Glcp-(1→

2 residues 2 residues

O-Acetyl groups were shown to be attached to O-6 of both the 3- and 4-substituted D-glucose residues and the low value for the optical rotation of the polysaccharide indicated that all the sugar residues are β-linked. If this polysaccharide is composed of repeating units, then the analyses imply that these should be octasaccharides. As hexasaccharides were previously the largest repeating units that had been conclusively demonstrated, the nature of this polysaccharide was of special interest. We have now studied this structure (Ref. 9) by subjecting the polysaccharide to a number of consecutive degradations, based upon oxidation - - β-elimination (Ref. 10).

The starting point for this sequence was the terminal D-glucopyranosyl residue, which has pyruvic acid acetalically linked to O-4 and O-6. When the carboxyl reduced and methylated polysaccharide (14) was subjected to acid hydrolysis under mild conditions, the acetalic linkages were cleaved without any cleavage of glycosidic bonds. On oxidation, using chlorine, dimethyl sulfoxide - triethyl amine (Ref. 11), carbonyl groups were introduced into the terminal residue (15). On treatment with base this residue was modified by β-elimination (possibly to 16) and eliminated on subsequent treatment with acid under mild conditions. Methylation analysis of the product (17) using trideuteriomethyl iodide as alkylating agent, demonstrated that the terminal was linked to O-3 of the penultimate residue.



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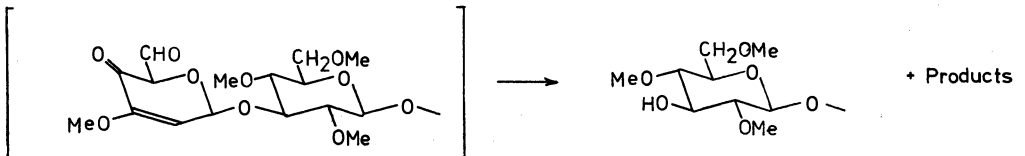
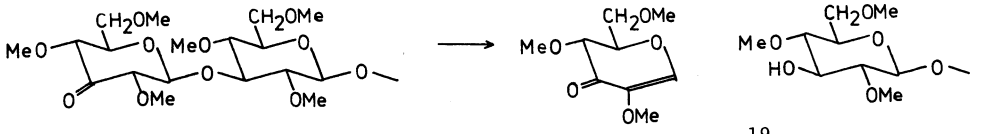


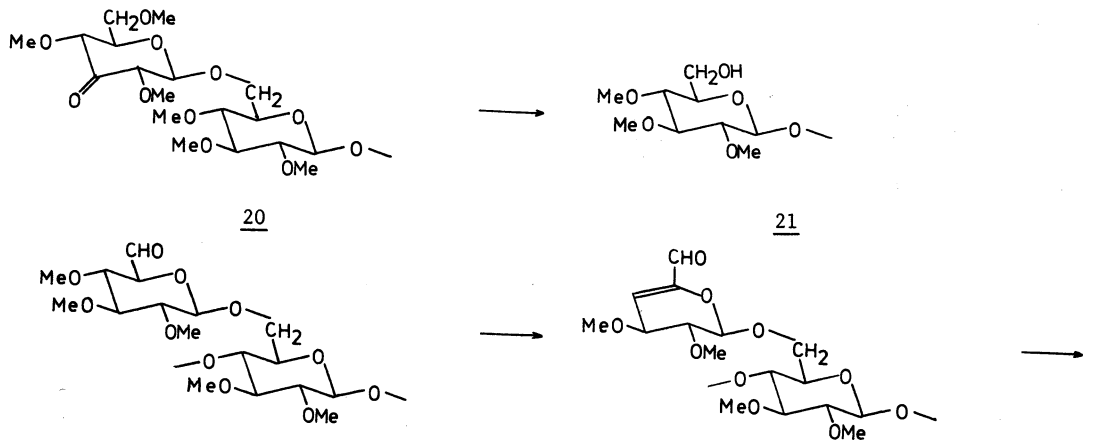
16 17

In the next step the modified polysaccharide (17) was oxidized to 18 and subjected to a second degradation. In this step the mild acid hydrolysis was not necessary since the terminal sugar was eliminated during the treatment with base. The product (19) again contained a free hydroxyl at C-3 of its new, terminal residue.



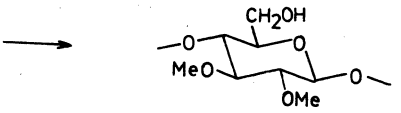
18 19

The third degradation, starting with the oxidation of 19 to 20, was analogous to the second, except that a hydroxyl at C-6 of the next residue (21) in the chain was exposed.



20 21

22 23



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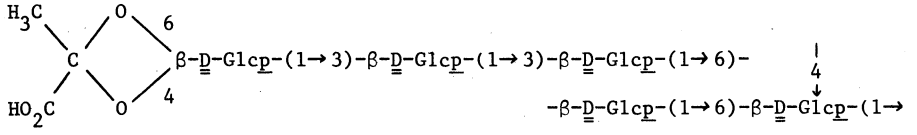
The modified polysaccharide 21 was oxidized to 22 and again subjected to degradation. A terminal hex-4-enodialdo-1,5-pyranosyl group (as in 23) should be formed on treatment with base, and eliminated during the mild acid hydrolysis. The product (24) was investigated as above, and 2,3,6-tri-O-methyl-D-glucose, with a trideuteriomethyl group at O-6, was obtained. Hence the terminal group removed was linked to O-6 of the branching D-glucose residue. So as the result of four consecutive degradations, the partial structure 25 has been established.



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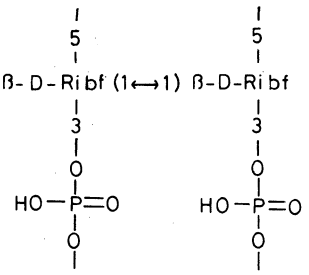
The courses of the first four degradations were relatively simple, as each separate step resulted in the elimination of a terminal residue. The fifth degradation, starting with the oxidation of 24, is more complicated and we are still working with it. The present results, however, strongly suggest that the capsular polysaccharide from Rhizobium meliloti has a regular structure and is composed of octasaccharide repeating units.

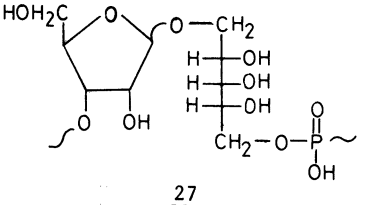
HAEMOPHILUS INFLUENZAE CAPSULAR ANTIGENS

Several of capsular antigens produced by different strains of Haemophilus influenzae contain phosphate and an unusual structure (26) has been proposed (Ref. 12) for the antigen from type b.



26

This is obviously inconsistent with the biosynthetic principles discussed above, and indeed a reinvestigation of the structure has proved it to be incorrect. A group from Lilly Research Laboratories showed the antigen to be composed of D-ribose, ribitol and phosphate (1:1:1) and determined its structure (27), mainly with the aid of ^{13}C n.m.r. (Ref. 13).



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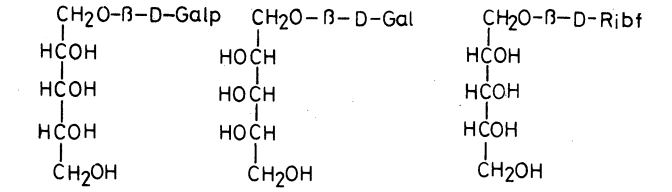
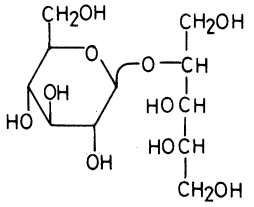
We arrived at the same structure and further proved that the β-D-ribofuranosyl residue is linked to O-1 of D-ribitol and the phosphate consequently to O-5 of the same residue, in agreement with the assumed biosynthetic route (Ref. 14). We had previously prepared the 1- and 5-O-β-D-galactopyranosyl-D-ribitols (28, 29) and determined their optical rotations both in water and in molybdate at pH 5.5 (Ref. 15). The alditol part, but not the glycosyl part of these compounds, complexes with molybdate (Ref. 16) and the strong shift in optical rotation is therefore determined by the structure of the former part. The β-D-ribofuranosyl-



28 29 30

-ribitol (30), prepared from the antigen, showed a strong negative shift, as did 1-O-β-D--galactopyranosyl-D-ribitol, thus demonstrating that the ribitol residue has the same configuration in the two substances.

The capsular antigen from H. influenzae type a proved to be composed of D-glucose, ribitol and phosphate (1:1:1) (Ref. 17). On dephosphorylation by treatment with base and then with alkaline phosphatase, 4-O-β-D-glucopyranosyl-D-ribitol (31) was obtained, and was indistinguishable from an authentic sample, synthesized by Baddiley and coworkers (Ref. 18). Again, for biosynthetic reasons, phosphate is expected to be linked to O-5 in the D-ribitol moiety.



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The ^{13}C n.m.r. spectrum of the antigen and of some relevant reference substances are given in Table 2. The signals given by 4-O-β-D-glucopyranosyl-D-ribitol could be assigned to the

TABLE 2. ^{13}C n.m.r. shifts of H. influenzae type a antigen and relevant reference substances

Compound	Chemical shift, in p.p.m. C-1	C-2	C-3	C-4	C-5	C-6	C'-1	C'-2	C'-3	C'-4	C'-5
Ribitol							63.2	72.9	72.9	72.9	63.2
Methyl β-D-glucopyranoside	103.9	73.8	76.5[a]	70.4	76.6[a]	61.5	57.9				
4-O-β-D-glucopyranosyl-D--ribitol	103.1	74.1	76.4[a]	70.4	76.6[a]	61.5[b]	63.5	72.7[c]	72.2[c]	81.7	61.1[b]
H.i. type a antigen	102.9	74.1	75.6[a,d]	74.8[e]	75.8[a,d]	61.4	63.5	72.6[c]	72.4[c]	80.1[f]	65.7[g]

a,b,c The assignments may be reversed

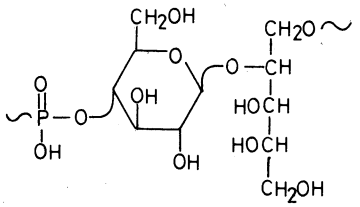
d Coupled signals, coupling constants not determined

e $^2J\ ^{31}P-^{13}C = 6$ Hz

f $^3J\ ^{31}P-^{13}C = 6.5$ Hz

g $^2J\ ^{31}P-^{13}C = 5$ Hz

different carbon atoms by the close correspondence of the chemical shifts with those of separate standards of methyl β-D-glucopyranoside and ribitol. The main difference was the expected downfield shift of C-4 in the ribitol moiety. Some ambiguities are of no consequence to the conclusions. In the antigen, strong downfield shifts are observed for C-5 in the D--ribitol moiety and C-4 in the D-glucose moiety. There is also $^{13}C - {}^{31}P$ coupling of the signals for C-3, C-4 and C-5 in the D-glucose moiety and for C-4 and C-5 in the D-ribitol moiety. Phosphate is consequently linked to O-4 of D-glucose and to O-5 of D-ribitol in the antigen (32).



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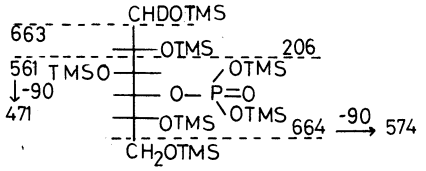
Sugar phosphates may be characterized by g.l.c.-m.s. after reduction to alditols with borodeuteride and subsequent trimethylsilylation. When the H. influenzae type a antigen was treated with M hydrochloric acid for 45 min at 100°C and the hydrolysate investigated by this method, ribitol-1-phosphate, ribitol-2-phosphate, D-glucitol-4-phosphate and D-glucitol--3-phospate were the phosphorylated derivatives obtained. The two latter compounds were monodeuterated at C-1. The origin of some typical fragments given by the trimethylsilylated D-glucitol-4-phosphate is indicated in the formula (33). The results therefore lend further



33

support to the proposed structure for the antigen (32), with phosphate linked to O-4 of D-glucose and O-5 of D-ribitol. The ribitol-2-phosphate and the D-glucitol-3-phosphate were probably formed by migration of the phosphate group during acid hydrolysis or borohydride reduction. This method for locating phosphate ester linkages in carbohydrates may be useful, especially for polymers with a complicated structure, for which ^{13}C n.m.r. gives only limited information.

Acknowledgement. - It is a pleasant duty to thank the members of our research group, listed in the bibliography, for their contributions to practical and theoretical aspects of this work. The work has been supported by the Swedish Natural Science Research Council, the Swedish Medical Science Research Council, Knut and Alice Wallenbergs Stiftelse, Harald Jeanssons Stiftelse and Stiftelsen Sigurd och Elsa Goljes Minne.

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