

MICROBIAL O-ANTIGENIC HEXOSAMINOGLYCANS

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Abstract - Lipopolysaccharides were isolated from dried cells of Shigella boydii, Escherichia coli and Pseudomonas aeruginosa species by extraction with hot, aqueous phenol. The antigens obtained were degraded with acetic acid to give O-specific polysaccharides which were purified by permeation chromatography on the Sephadex G-50 column. The isolated polysaccharides were proved to be complex hexosaminoglycans composed of oligosaccharide repeating units containing various number of amino sugar residues. The C-n.m.r. spectroscopy, methylation analysis and selective cleavage were the principal methods used for structural analysis. Immunological properties of the specific hexosaminoglycans and modern methodology of the structural polysaccharide analysis were discussed.

INTRODUCTION

The upper layer of the outer membrane of gram-negative bacteria is composed of lipids, proteins and lipopolysaccharides molecules which are conjugated by different types of linkages to form intermolecular complexes. The exposed components of these complexes are antigens determining the immunological specificity of microbial cells. The most important constituents of these complexes are thought to be lipopolysaccharides which play a role of endotoxins (Ref. 1), specific receptors for viruses (Ref. 2), and somatic antigens (Ref. 3). Knowledge of the chemical structure of lipopolysaccharides is of great significance for understanding of molecular principles of their biological activities. Moreover, chemical studies on lipopolysaccharides, which are the molecules with enormously complicated structures, are important for chemistry itself as a stimulus in creation of new methods, approaches and ideas. Our structural investigations on the O-specific polysaccharides isolated from Shigella and P.aeruginosa lipopolysaccharides were inspired by the brilliant works of O.Westphal and O.Lüderitz on Salmonella lipopolysaccharides. We have chosen Shigella and P.aeruginosa for three reasons: 1. They are pathogenic for man; 2. They make rather small groups of serologically typed species and this is convenient for comparative immunochemical research; 3. The chemical data on their lipopolysaccharides were scanty.

There are about 40 different serotypes of Shigella, which may be divided into four main groups: Group A (Sh.dysenteriae), Group B (Sh.flexneri), Group C (Sh boydii) and Group D (Sh.sonnei). Earlier in this century dysentery caused by Sh.dysenteriae reached the pandemic proportions with high mortality rates. Later Sh.dysenteriae was replaced by Sh.flexneri and Sh.sonnei, less virulent organisms. Bacillary dysentery is primary a disease of children from 6 months to 10 years of age and is particularly prevalent among children confined to institutions. Second attacks are not uncommon, particularly under conditions of poor sanitation. There is a great need for an efficient vaccination scheme since a great percentage of the world's population lives under conditions that favour the outbreak of epidemics of dysentery. For a long time attempts to immunize man against Shigella had been unsuccessful. However, in 1965, a big group of volunteers was administered orally with a streptomycin-dependent strain of Sh.flexneri to prove that immunological protection is possible. The results obtained were of great interest, since they showed that oral vaccination provided effective protection against infection and indicated that protective antibodies were directed against the type-specific O-somatic antigen, i.e. lipopolysaccharide (Ref. 4).

P.aeruginosa is a potentially dangerous pathogen which causes severe and often lethal infections in debilitated patients whose immunological system has been weakened by trauma arising from severe burns or from surgery. These infections are especially troublesome if they appear in hospitals. The use of

chemotherapy in treating such infections appeared to be ineffective due to resistance of the microorganism to commercial antibiotics, while an immunological approach seems promising. Indeed, heptavalent vaccine composed of individual somatic antigens isolated from cells of seven immunoprotective types was used in United States for prophylactic against invasive infection. The administration of vaccine afforded a dramatic decrease in the mortality rate from *P.aeruginosa* sepsis (Ref. 5).

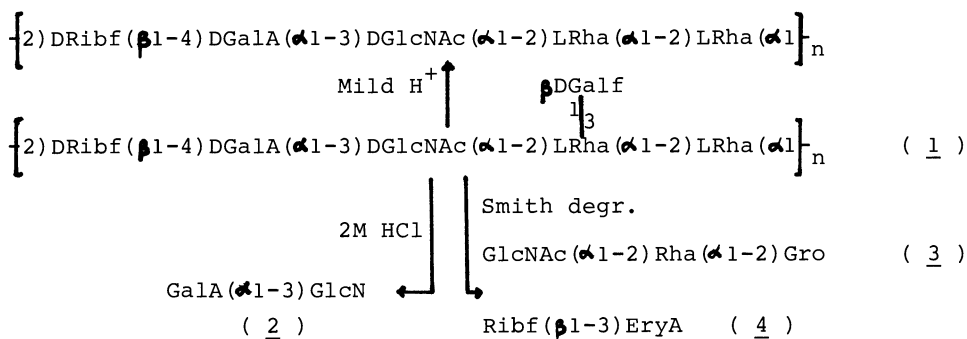
The attempts at chemical analysis of the lipopolysaccharides from *Shigella* and *P.aeruginosa* have been earlier undertaken by different groups of scientists. At present, the chemical structures of all the O-specific polysaccharides from *Sh.dysenteriae* (Ref. 6), *Sh.flexneri* (Ref. 7) and *Sh.sonnei* (Ref.8) are elucidated. This success was reached mainly due to developing an effective approach based on the combined use of physical (nuclear magnetic resonance spectroscopy, infrared spectroscopy and electrophoresis) and chemical (selective cleavage) methods. The O-specific polysaccharides of *Sh.boydii* are intensively investigated at present in our laboratory, and the structures of polysaccharides from several serologically related *Sh.boydii* and *E.coli* species will be discussed below. It is interesting to note that certain strains of *E.coli* cause a disease almost indistinguishable from shigellosis and these strains possess somatic antigens chemically identical to those of *Shigella* (Ref. 9 & 10). In comparison with *Shigella*, less is known about the structures of the O-specific polysaccharides from *P.aeruginosa* because these investigations turned out to be extremely difficult due to presence of unknown amino sugars, and here we report on the first structural data obtained in our group.

STRUCTURAL ASPECTS

O-Specific polysaccharides of *Sh.boydii* and serologically related *E.coli*

Sh.boydii type 2 (Ref. 11). The characteristics presented by physical methods distinctly indicated that polysaccharide was made up of the repeating hexasaccharide units composed of following types of sugars: uronic acid, N-acetylamino sugar, two 6-deoxyhexoses and two ordinary neutral sugars one of which was pentose. It was also evident that two sugar residues were in the furanose form. The polysaccharide was readily hydrolysed into monosaccharides among which were identified D-galactose, D-ribose, L-rhamnose, D-glucosamine and D-glucuronic acid in the ratio 1:1:2:0.7:0.7. Diminished content of two latter sugars indicated that glucosamine was retained by glucuronic acid, and this disaccharide 2 was isolated from the hydrolysate. As it followed from the results of methylation analysis, the *Sh.boydii* type 2 specific polysaccharide was branched at one of two rhamnose residues, galactofuranose being the side chain terminus. The occurrence of terminal furanose residue suggested the application of partial acid hydrolysis, which was performed using 0.025 M oxalic acid to give a modified linear polysaccharide. Methylation analysis as well as comparison of C-n.m.r. spectra of both polysaccharides proved independently that the terminal galactofuranose residue in the polysaccharide was attached to position 3 of the branched rhamnopyranose residue. The subsequent structural data on the polysaccharide, whose repeating unit is given on the Scheme 1, were obtained with the use of Smith degradation.

Scheme 1 .

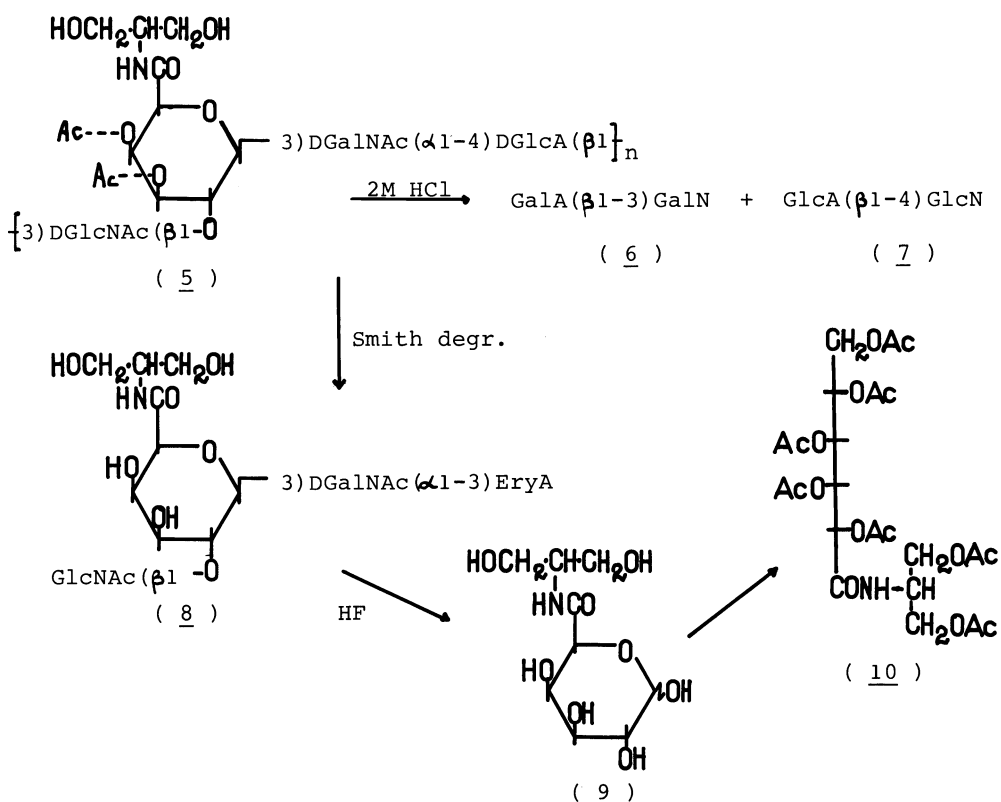


Smith degradation of the polysaccharide resulted in formation of two fragments i.e. disaccharide 3 composed of N-acetyl-glucosamine, rhamnose and glycerol, and glycoside 4 identified as ribofuranosyl(1-3)erythronic acid. Isolation of oligosaccharides 3 and 4 proved unequivocally the sequence of monosaccharide residues in the main chain of the polysaccharide and the attachment of the galactofuranose side chain to the rhamnose residue adjacent to N-acetylglucosamine. The configurations of glycosidic linkages followed from the detailed analysis of the C-n.m.r. spectrum of the polysaccharide. The assignment of

the signals in the spectrum was in a good agreement with the structure 1 of the polysaccharide.

Sh.boydii type 8 (Ref. 12). As it followed from the data obtained by physical methods, the O-specific polysaccharide from Sh.boydii type 8 was an acidic hexosaminoglycan with tetrasaccharide repeating unit carrying an unusual substituent. Indeed, the total number of signals in the C-n.m.r. spectrum exceeded by three the number calculated for four hexoside residues and, on the other hand, the signals for two N-acetyl groups but three carbon atoms linked with nitrogen were present in the spectrum. Drastic acid hydrolysis (6M hydrochloric acid) of the polysaccharide followed by amino sugar analysis led to identification of D-galactosamine, D-glucosamine and 2-aminopropanediol-1,3 in the equimolar ratio. At the same time, conventional hydrolysis with 2M hydrochloric acid resulted mainly in formation of two disaccharides 6 and 7 each composed of uronic acid and amino sugar as it is shown on the Scheme 2. The structures of these disaccharides were established by routine methods. The sequence of monosaccharide residues in the repeating unit became evident from the structures of disaccharides 6 and 7, and it was, in addition, independently confirmed by results of Smith degradation which led to trisaccharide fragment 8.

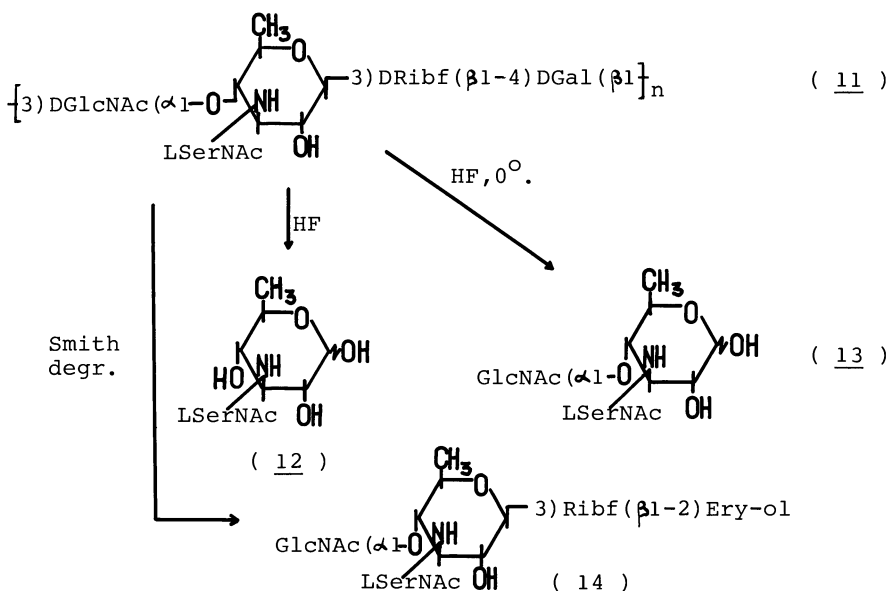
Scheme 2 .



The substitution patterns in the polysaccharide and trisaccharide 8 were determined by methylation analysis. Moreover, the positions of two non-stoichiometrical O-acetyl groups, which were present in the repeating unit, were also evident from methylation analysis providing that methylation was performed in a short time (15 min.) insufficient for the de-O-acetylation reaction to occur. Unusual amide 9 of galacturonic acid was obtained from trisaccharide 8 after its cleavage with liquid hydrogen fluoride. The structure of amide 9 was proved both by conversion into acetylated alditol 10 followed by gas chromatography/mass-spectrometry analysis and by the synthesis starting from galacturonic acid and 2-aminopropanediol-1,3. The configurations of glycosidic linkages were evident from the C-n.m.r. spectrum of the de-O-acetylated polysaccharide. The presence of two non-stoichiometrical O-acetyl groups on the galacturonic acid residue is thought to be a result of migration, which could occur at the stage of lipopolysaccharide isolation or of "degraded polysaccharide" preparation.

E.coli O:114 (Ref. 13). E.coli O:114 is known to exhibit cross-reaction with Sh.boydii type 8, and it was logical to compare the structures of their O-specific polysaccharides. The first distinction in structures became evident after electrophoretic analysis when it was shown that the specific polysaccharide from E.coli appeared to be neutral hexosaminoglycan. The first order analysis of the C-n.m.r. spectrum of the polysaccharide revealed four anomeric carbon atoms, one of which belonged to furanoside residue. However, there were five terminal carbon atoms (four hydroxy-methyl groups and one C-methyl group), and two N-acetyl groups but three carbon atoms linked with nitrogen. This indicated the repeating unit of the polysaccharide was composed of four monosaccharide residues and one unknown constituent. Drastic acid hydrolysis (4M hydrochloric acid) of the polysaccharide followed by analysis with the use of amino acid analyser led to identification of D-glucosamine, 3-amino-3,6-dideoxy-D-glucose and L-serine in the ratio 1:1:1. The conventional hydrolysis (2M hydrochloric acid) resulted in identification of D-galactose, D-ribose, D-glucosamine and new amino component identified as 3-(L-seryl)amino-3,6-dideoxy-D-glucose. Results of methylation analysis showed the ribose residue was in furanose form and the substitution patterns were favourable for Smith degradation because galactopyranose residue was substituted in position 4. The structure of the polysaccharide was deduced from the results of two solvolyses with liquid hydrogen fluoride and Smith degradation as it is shown on the Scheme 3.

Scheme 3 .

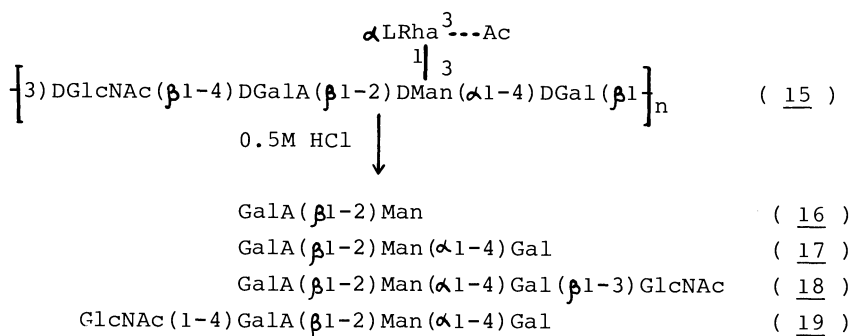


The configurations of glycosidic linkages in the polysaccharide followed from the C-n.m.r. spectrum, the signals being successfully assigned with the help of spectral data for compounds 12 13 and 14. The presented data unequivocally indicated that the specific polysaccharides from E.coli O:114 and Sh.boydii type 8 have completely different structures and scarcely could be responsible for the serological relationship mentioned above.

Sh.boydii type 12 (Ref. 14). In the C-n.m.r. spectrum of the polysaccharide

there were observed the characteristic signals for five anomeric carbon atoms and five terminal carbon atoms (three hydroxy-methyl groups, one C-methyl group of 6-deoxysugar and one carboxyl group) as well as the signals of one N-acetyl group and two non-stoichiometric O-acetyl groups attached to different monosaccharide residues. The polysaccharide was readily hydrolysed with 2M hydrochloric acid to give L-rhamnose, D-mannose, D-galactose, N-acetyl-D-glucosamine and D-glucuronic acid in the equimolar ratios. The data obtained indicated the repeating unit of the polysaccharide was pentasaccharide. As it followed from results of methylation analysis, the polysaccharide had a branched structure with the side chains terminated by rhamnose residues, and mannose being the branching point. The sequence of monosaccharide residues in the polysaccharide was evident from the results of partial acid hydrolysis which led to formation of a set of oligosaccharides as is shown on Scheme 4.

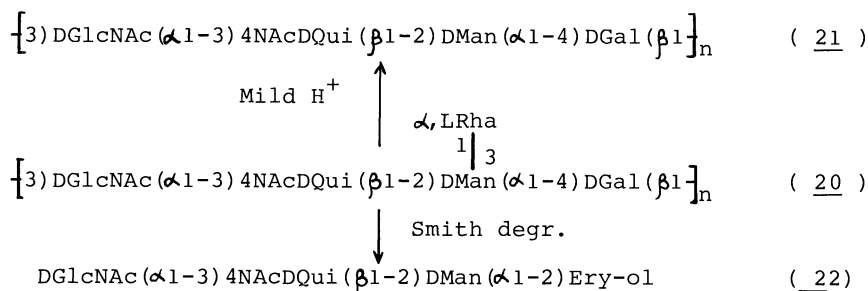
Scheme 4 .



At present, location of the second O-acetyl group is in progress. It is noteworthy that C-n.m.r. spectrum of the de-O-acetylated polysaccharide is to a certain extent analogous to that of the polysaccharide from *E.coli* O:7 thus indicating the structural relationship between these polysaccharides from two cross-reacted bacteria.

E.coli O:7 (Ref. 15). Contrary to the previous polysaccharide, the O-specific polysaccharide from *E.coli* O:7 was neutral hexosaminoglycan. Its pentasaccharide repeating unit was composed of two N-acetylamino sugars, two hexoses and one 6-deoxyhexose residue. The comparison of C-n.m.r. spectrum of the polysaccharide with that of the polysaccharide from *Sh.boydii* type 12 revealed a group of identical signals belonging to the carbon atoms of rhamnose, mannose and galactose residues. The polysaccharide was subjected to solvolysis with liquid hydrogen fluoride to give equimolar amounts of L-rhamnose, D-mannose, D-galactose, N-acetyl-D-glucosamine and 4-acetamido-4,6-dideoxy-D-glucose. As it followed from results of methylation analysis, the polysaccharide was branched, and the substitution patterns were analogous to those of the polysaccharide from *Sh.boydii* type 12. The terminal rhamnose residue was attached to position 3 of the disubstituted mannose residue, and this was proved by conversion of the branched polymer into a linear one by means of mild acid hydrolysis. To determine the sequence of monosaccharide residues in the main chain, the polysaccharide was subjected to Smith degradation to give a sole trisaccharide fragment (22), as it is shown on the Scheme 5.

Scheme 5 .

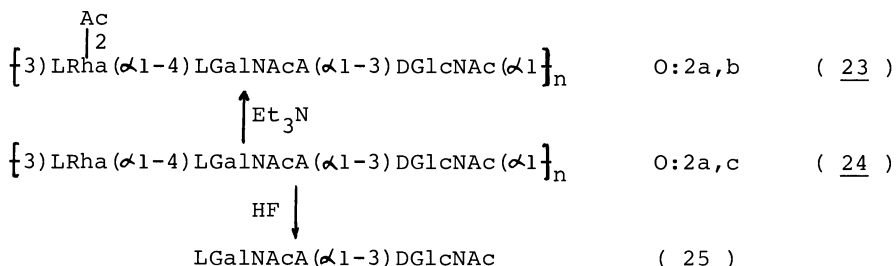


The signals in the C-n.m.r. spectrum of the polysaccharide were assigned with the use of spectral data for trisaccharide derivative 22 and linear polysaccharide 21. The present data demonstrate the structural relationship between the specific polysaccharides from *E. coli* O:7 and *Sh.boydii* type 12.

O-Specific polysaccharides from *Pseudomonas aeruginosa*
P.aeruginosa O:2 (Ref. 16). According to Lanyi classification, the serological group O:2 of *P.aeruginosa* comprises two serotypes 2a,b and 2a,c. The O-specific polysaccharides isolated from both serotypes appeared to be acidic hexosaminoglycans composed of L-rhamnose, N-acetyl-D-quinovosamine and N-acetyl-L-galactosaminuronic acid residues. To avoid erroneous results, the polysaccharides were carboxyl-reduced prior to acid hydrolysis. The spectral data indicated that both polysaccharides were identical except that rhamnose residue in the specific polysaccharide O:2a,b was substituted in position 2 by an acetyl group. Alkaline saponification readily converted the polysaccharide O:2a,b into the polysaccharide O:2a,c undistinguishable from the original O:2a,c hapten

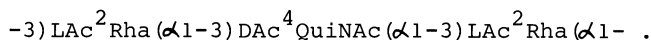
by serological and spectral characteristics. The structures of O:2 specific polysaccharides are presented on the Scheme 6.

Scheme 6 .



The substitution types of the monosaccharide residues were determined by methylation analysis of the carboxyl-reduced polysaccharide, and the sequence of units in the linear chain of the O:2a,c polysaccharide followed from the structure of disaccharide 25 obtained by a selective solvolysis with hydrogen fluoride. The configurations of glycosidic linkages were evident from the spectral data.

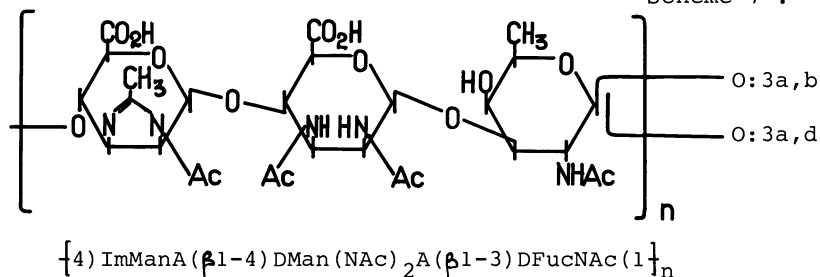
Recently (Ref.17), structural data on the O-specific polysaccharide of *P.aeruginosa* immunotype 5 have been published. The serological properties of immunotype 5 are covered by the serogroup O:2. The authors found the repeating unit of the polysaccharide to be composed of two rhamnose and one N-acetylquinovosamine residues, and the following structure was assigned to it:



The proposed structure differs dramatically from the structure established in our investigation. Assuming the serological relationship between serogroup O:2 and immunotype 5, one would expect strong similarity, if not identity, in the chemical structures of the corresponding O-specific polysaccharides. However, this appears not to be so, and no sensible discussion of this situation can be made until direct comparison of the antigens in question has been performed. Nevertheless, it should be noted that the content of rhamnose and quinovosamine in the ratio 2:1 could be easily obtained for both O:2 polysaccharides when acid hydrolysis followed by g.l.c. techniques are used for sugar quantitation. We proved that galactosamiuronic acid retains quinovosamine residue, and disregard of this fact may cause the erroneous interpretation of the results obtained.

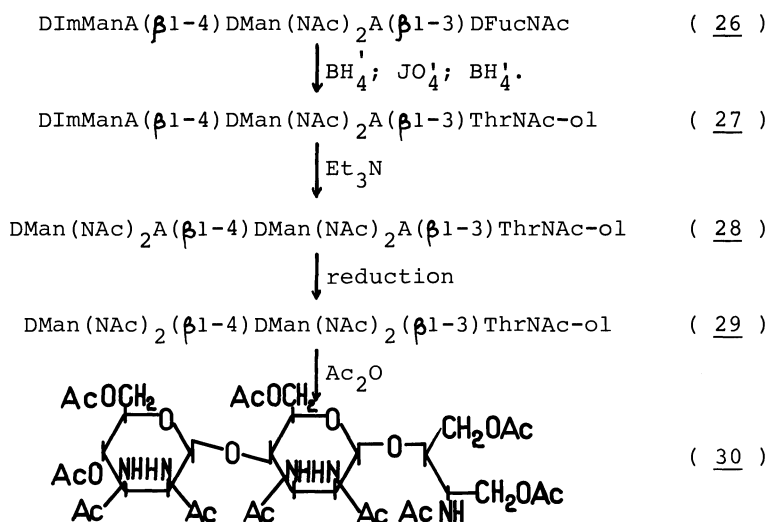
P.aeruginosa O:3 (Ref. 18 & 19). According to Lanyi classification the O:3 group of *P.aeruginosa* is divided into five serotypes: O:3a,b ; O:3a,c ; O:3a,d ; O:3a,d,e and O:3a,f. The structures of the first four O-specific polysaccharides will be discussed below. The chemical investigation of these polysaccharides was hampered by the unusual sugar composition which was impossible to determine with the use of conventional methods based on acid hydrolysis. Indeed, after acid hydrolysis of these polysaccharides we were able to identify fucosamine only and in the quantity not exceeding 5%, the other products being oligomers resistant even to drastic conditions. Therefore, we abandoned the determination of the sugar composition as the stage preceding the structural analysis and concentrated on the selective cleavage of these polysaccharides into oligosaccharide fragments composed of unidentified sugars to subject them to investigation by combination of chemical and spectral methods. Firstly, this approach was applied to the O-specific polysaccharide from *P.aeruginosa* O:6 (see below) and afterwards to the polysaccharides of O:3 group. The structures of the O:3a,b and O:3a,d polysaccharides are depicted on Scheme 7.

Scheme 7 .



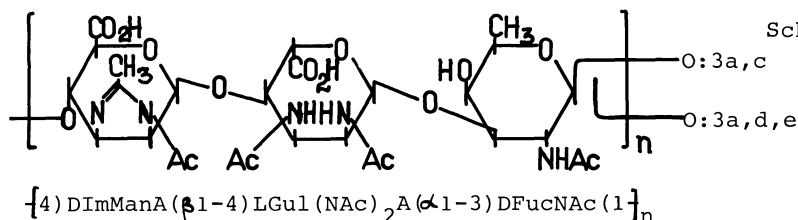
The preliminary chemical as well as spectral and electrophoretical data indicated that both polysaccharides were acidic hexosaminoglycans composed of trisaccharide repeating units made up of N-acetylfucosamine and two new acidic diamino sugars the structures of which were difficult to predict. The structures of these uncommon sugars were elucidated in the course of structural analysis of the polysaccharides in the following way. We discovered that both O:3a,b and O:3a,d specific polysaccharides gave upon treatment with liquid hydrogen fluoride the same trisaccharide (26) with N-acetylfucosamine residue at the reducing end. The spectral data indicated that trisaccharide 26 comprised all the sugars and substituents that were in the original polysaccharide. Further, trisaccharide 26 was subjected to successive chemical transformations aimed at the simplification of the molecule by means of destruction of fucosamine residue and unification of the rest residues as it is shown on Scheme 8. Simultaneously the spectral data were accumulated for each derivative thus prepared, and this was done until the compound was obtained with a spectrum of unequivocal interpretation.

Scheme 8 .



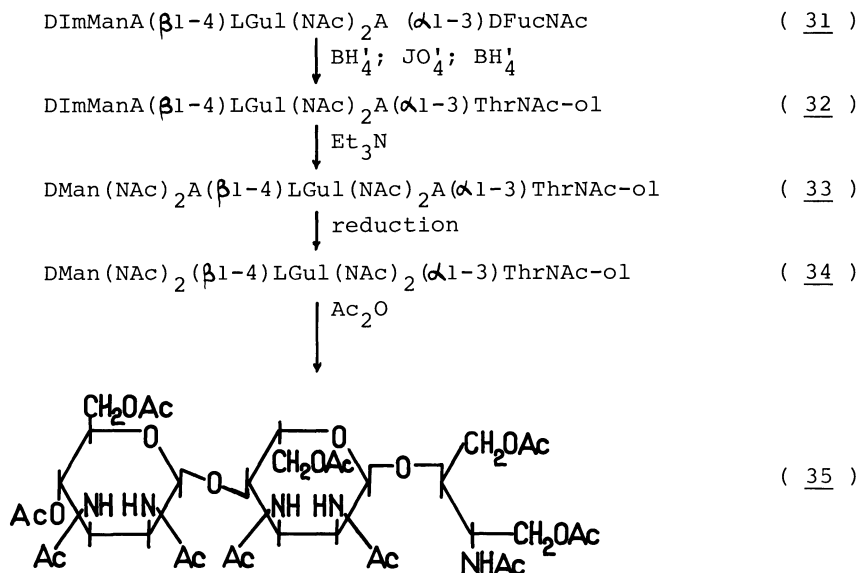
Trisaccharide 26 was modified at the reducing N-acetylfucosamine residue into derivative 27 by successive borohydride reduction, periodate oxidation and borohydride reduction again, then 2-imidazoline cycle was opened up by triethyl amine treatment to give 28. Afterwards the carboxylic groups of uronic acid residues were reduced by carbodiimide-associated borohydride treatment into primary hydroxy groups. Acetylation of 29 led to derivative 30 with spectral characteristic suitable for interpretation. Analyses of the H and C-n.m.r. spectra and mass-spectrum of this acetate have shown the presence of two 2,3-diacetamido-2,3-dideoxy-mannopyranose residues. This conclusion was confirmed by the isolation of diamino hexose after hydrolysis of 29 and its identification by direct comparison with the authentic, synthetic sample. Then, the retrospective comparison of the chemical behaviour and the spectral characteristics of all the oligosaccharide derivatives, moving backward from compound 29 to trisaccharide 26 led us to conclusion that terminal diamino sugar residue in trisaccharide 26 was in the form of 1-acetyl-2-methyl-2-imidazoline derivative. This bicyclic sugar and the parent 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid have been discovered in Nature for the first time. All signals in the C-n.m.r. spectrum of trisaccharide 26 were assigned, and these data along with the results of methylation analysis proved the mode of conjunction of the trisaccharide repeating units into linear chains. The polysaccharide structures differed by the configuration of fucosamine glycosidic linkages and this proved to be sufficient for the serological difference.

The structures of the second pair of specific polysaccharides from *P.aeruginosa* O:3a,c and O:3a,d,e types are depicted on the Scheme 9. Both polysaccharides were analogously composed of the trisaccharide repeating units, and one could easily notice that these structures were doubtless related to those for sero-types O:3a,b and O:3a,d.



Consequently, the spectral characteristics of these polysaccharides provided us with familiar information: the trisaccharide repeating unit was composed of a known N-acetyl-D-fucosamine and imidazoline derivative residues along with the residue of another diamino hexuronic acid. Therefore, the approach to the elucidation of the polysaccharide structures was as already described above. Both polysaccharides were treated with hydrogen fluoride to split selectively the N-acetylfucosaminide linkages. Solvolyses resulted in formation of trisaccharide 31 which was further subjected to successive chemical modifications, shown on the Scheme 10, with parallel spectral investigation.

Scheme 10.

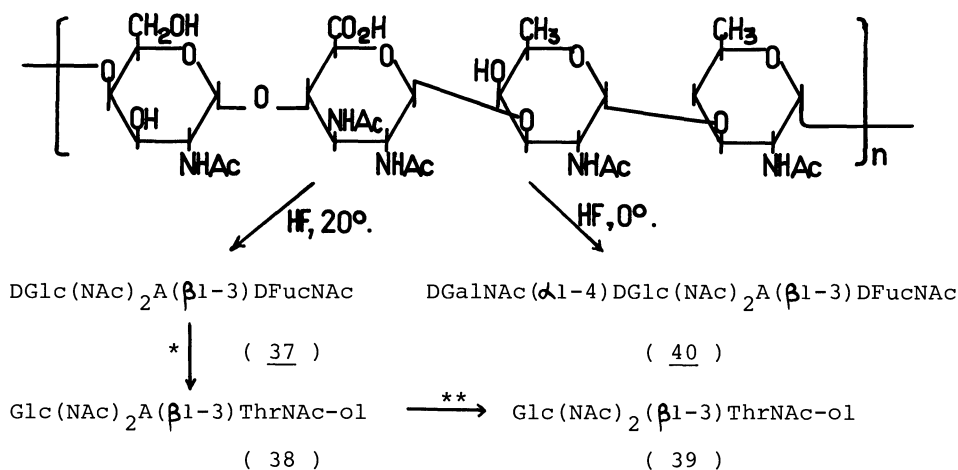


The spectral characteristics of the acetylated derivative 35 enabled us to identify the terminal residue which was already known as 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid. The successive assignment of the signals for 2-acetamido-2-deoxythreitol and 2,3-diacetamido-2,3-dideoxymannuronic acid residues allowed to select from the spectrum of the derivative 34 the signals of unknown diacetamido dideoxyhexose residue. The up-field shift of the resonance of C5 atom indicated that new hexose was linked with α -glycosidic bond. The absence of the model diacetamido dideoxy hexoses hampered the assignment of the signals, and, therefore, we tried to calculate theoretically the chemical shifts for glycosides of eight isomeric 2,3-diacetamido-2,3-dideoxyhexoses. The resonances calculated for methyl 2,3-diacetamido-2,3-dideoxy-L-guloside coincided with those of the unknown sugar residue. Moreover, the independent synthesis of the authentic sample of 2,3-diacetamido-2,3-dideoxy-L-gulose and investigation of its C-n.m.r. spectrum confirmed the correctness of this supposition. The complete interpretation of the C-n.m.r. spectrum of the derivative 34 promoted the successive interpretation of the spectra of oligosaccharides 33, 32 and 31, and finally the spectra of both specific polysaccharides. The results of combined chemical and spectral investigations carried out on four O-specific polysaccharides from *P.aeruginosa* O:3 group demonstrated clearly the structural relationship of the antigens causing the serological relationship of the corresponding microbial species. The immunological aspect of this problem will be discussed below.

P.aeruginosa O:6 (Ref. 20 & 21). The O:6 specific polysaccharide proved to be hexosaminoglycan made up exclusively of different types of amino sugars, one of them an unknown diamino sugar. An attempt to determine the monosaccharide

composition of the polysaccharide using complete acid hydrolysis was not very successful, because it resulted in complete release of D-galactosamine and D-quinovosamine only, whereas D-fucosamine was retained to a considerable extent by acidic diamino sugar. The structure of this novel sugar and the sequence of monosaccharide residues were elucidated simultaneously in the course of structural analysis of oligosaccharide fragments resulting from the polysaccharide after its solvolytic depolymerisation with liquid HF,

Scheme 11 .

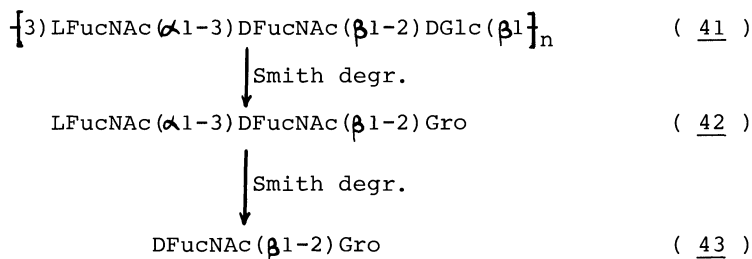


* stands for successive borohydride reduction, periodate oxidation and borohydride reduction; ** stands for carbodiimide-associated reduction with borohydride.

Depending on conditions, liquid hydrogen fluoride cleaved different glycosidic linkages to give disaccharide 37 and trisaccharide 40. Subsequent chemical transformations of disaccharide 37 resulted in the glycosides 38 and 39 with structures established by spectral methods and finally proved by conversion into 2,3-diacetamido-2,3-dideoxy-D-glucose, isolated in crystalline form and compared with the authentic sample. From the structure of trisaccharide 40 in combination with the results of methylation analysis, the sequence of monosaccharide residues in the repeating unit of the polysaccharide and the types of their substitution were ascertained. The C-n.m.r. spectrum of the polysaccharide was fully interpreted with the use of spectral characteristics for oligosaccharides 37, 40 and appropriate model glycosides.

P. aeruginosa O:7 (Ref. 22). Group O:7 comprises two serotypes O:7a,b and O:7a,c. However, it has been shown that strains of each serotype produce identical lipopolysaccharides. The specific polysaccharides prepared from the corresponding lipopolysaccharides were composed of D-glucose, N-acetyl-D-fucosamine and N-acetyl-L-fucosamine in the ratio 1:1:1. These data were in agreement with the spectral characteristics. The sequence of monosaccharide residues in the linear polysaccharide chain was determined after two successive Smith degradations, as it is shown in Scheme 12.

Scheme 12



Acid hydrolysis of oligosaccharide 42 resulted in formation of racemic fucosamine, whereas pure D-fucosamine was isolated from the hydrolysate of glycoside 43. The spectral data of glycoside 43 and oligosaccharide 42 were used for

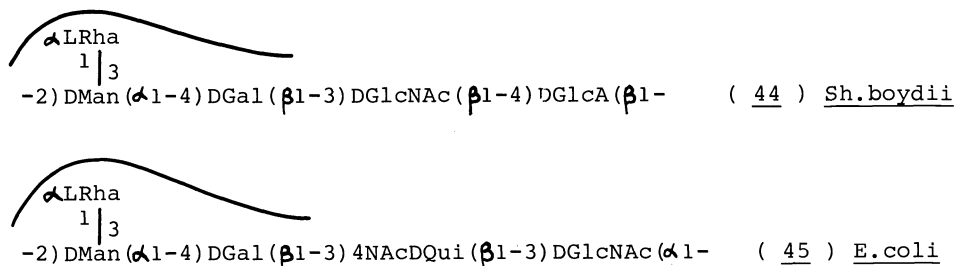
complete assignment of all the signals in the spectrum of the polysaccharide.

IMMUNOLOGICAL ASPECTS

Structures and serological factors

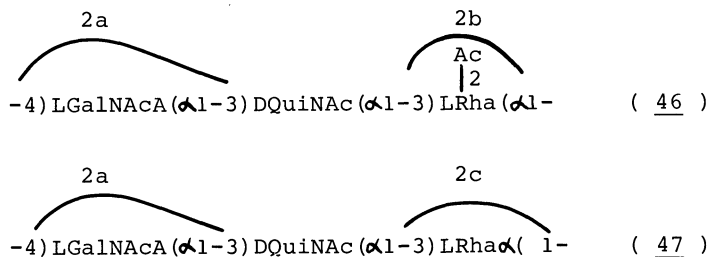
The main purpose of immunological investigations on microbial polysaccharides is, as a rule, to establish the correlation between the immunological specificity of microbial cells and the chemical structures of the corresponding mucilaginous, capsular and somatic antigens occurring on their surface. A number of chemical structures of the O-specific polysaccharides of *Sh.boydii*, *E.coli* and *P.aeruginosa* was considered in the previous section. Among these microbial species there were serologically insulated, e.g. *Sh.boydii* type 2 and *P.aeruginosa* O:6, and serologically related types, and the latter used to manifest similarity to form groups within one genera of microbes or just exhibited an occasional likeness. Thus, the chemical data obtained in this research are considered to provide an opportunity to support the earlier established serological relationship between certain microbial species in terms of structural similarity of their specific polysaccharides. At the beginning, cases are considered where chemical and serological data were in agreement. As it was mentioned above, the cells of *Sh.boydii* type 12 and *E.coli* O:7 strongly cross-reacted with the appropriate antisera. Nevertheless, the thorough comparison of the structures of their O-specific polysaccharides (formulae 44 and 45), which are distinctly different, easily reveals that three of five monosaccharide residues in each repeating unit form the identical structural assembly marked by arc-line on the Scheme 13.

Scheme 13 .



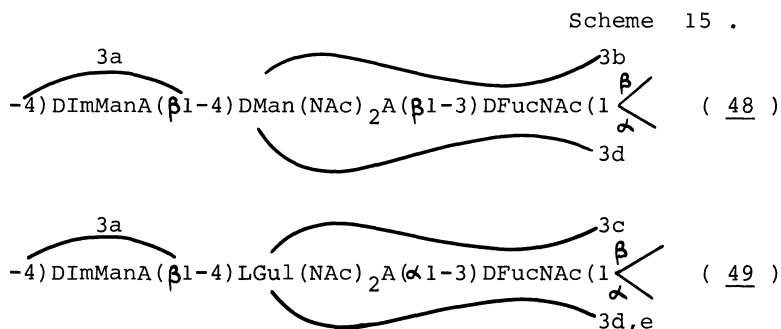
The common structural fragments comprise the terminal sugar residues of the side chain and the 2-O-substituted sugar residue of the main chain, these types of residues known to be the immunodominant components of the antigenic polysaccharides. Thus, the serological relationship of *Sh.boydii* type 12 and *E.coli* O:7 is evidently conditioned by the similar O-specific polysaccharide chain fragments in their somatic antigens. Analogous conclusion seemed to be fair with *P.aeruginosa* O;2 serogroup. The serological relationship of O:2a,b and O:2a,c types expressed by factor 2a is thought to be caused by common structural features in their O-specific polysaccharides, as it is depicted on the Scheme 14.

Scheme 14 .



Since the carboxyl-reduced polysaccharides were deprived of the O:2 specificity, one could consider the 2-acetamido-2-deoxy- α -L-galacturonic acid residue to be the immunodominant sugar responsible for factor 2a in both antigens. Serological factor 2b is associated with 2-O-acetyl- α -L-rhamnose residue and factor 2c with non-acetylated rhamnose residue, since alkaline saponification of O-acetyl groups in the O:2a,b polysaccharide resulted in appearance of O:2

a,c specificity, i.e. the conversion of serological factors has occurred. Thus, division of the *P.aeruginosa* O:2 group in Lanyi classification into two serotypes is confirmed objectively by chemical data. Different picture was observed in the studies on *P.aeruginosa* O:7a,b and O:7 a,c antigens which revealed no structural differences in the O-specific chains of the lipopolysaccharides, thus questioning the serological difference observed earlier. The impressive results, revealing the structural basis for specificity factors for a comparatively big group of bacteria, were obtained in the course of investigation on *P.aeruginosa* O:3 specific polysaccharides and their structures are presented on the Scheme 15.



The factor O:3a, common for all four serotypes, is undoubtedly caused by the residue of 2,3-(1-acetyl-2-methyl-2-imidazolino-5,4)-2,3-dideoxy- β -D-mannuronic acid for this residue is the only common structural fragment in each specific polysaccharide. The type specific factors, which reflect the difference in specificities, were conditioned by variations in two centres of the trisaccharide repeating unit, i.e. in configurations of the anomeric carbon atom of N-acetyl-D-fucosamine residue and the fifth carbon atom of 2,3-diacetamido-2,3-dideoxyhexuronic acid residue.

The assignments of serological factors to the strictly defined structural fragments of the O-specific polysaccharides, that became possible as a result of the accomplished comparative structural analysis of the antigens in question, must be considered as tentative ones and verified independently in the serological tests with the corresponding oligosaccharides and artificial antigens carrying these oligosaccharide determinants.

Discrepancy between chemical and serological data. The absence of chemical relationship between O-specific polysaccharides was observed in the case of *Sh.boydii* type 8 and *E.coli* O:114. The cells were known to cross-react, whereas their O-specific polysaccharides were composed of different sugars and non-carbohydrate constituents. This conflicting situation requires further thorough investigation.

METHODOLOGICAL ASPECTS

Tactics of structural analysis

Role of physical methods. In course of chemical investigation on the specific hexosaminoglycans from *Shigella*, *E.coli* and *P.aeruginosa* we encountered a number of experimental difficulties which were caused by uncommon sugar composition of the polysaccharides. The polysaccharides were made up of all types of monosaccharides and, from the other hand, these sugars were not always possible to detect with the help of acid hydrolysis followed by conventional analyses with chromatographic methods. In other words, a situation arose where incompleteness of data on the sugar composition hampered the structural analysis of the polysaccharides. The problem was resolved with the use of alternative approach the idea of which was to gain an essential structural information about polysaccharides by means of physical methods. This approach proved to be successful and was applied to the polysaccharides as it was briefly described in the previous sections.

The first and the simplest method to use was paper electrophoresis. The electrophoretic control indicated the presence of acidic constituents in the polysaccharide. The acidic sugars readily escape detection being inconvenient for analyses by gas liquid chromatography and ion-exchange chromatography in borate buffers. Moreover, they are able to retain adjacent sugars, especially if the latter are hexosamines. Disregard of these properties will undoubtedly result in erroneous conclusion on the polysaccharide structure. Thus, the evident electrophoretic mobility of hexosaminoglycan must be considered as a

warning against possible complications which could be prevented by the identification of acidic component. The most important information is evidently provided by spectral methods, i.e. H and C nuclear magnetic resonance spectroscopy of high resolution. In our research both methods were used intensively, C-n.m.r. being more informative. The signals in C-n.m.r. spectrum of hexosaminoglycan are known to distribute in groups each of which occupies strictly defined regions. These regions are characteristic for resonances of definite carbon atoms in different amino sugar residues and their substituents. The typical resonance regions useful for the first order analysis of the hexosaminoglycan spectra are shown on Fig. 1.

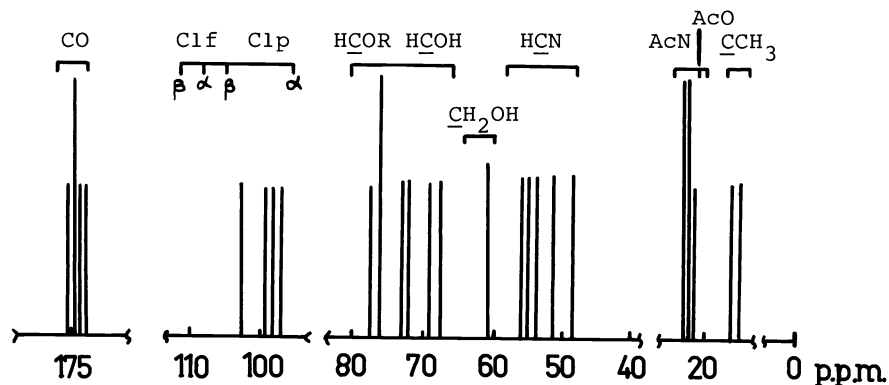


Fig. 1. ^{13}C -n.m.r. spectrum of the *P.aeruginosa* O:6 specific polysaccharide. On the top are given the characteristic regions for resonances of carbon atoms belonging to different monosaccharide residues in hexosaminoglycans.

As a rule, we started the first order analysis of the spectra by considering the resonance region for anomeric carbon atoms. This provided us with the information about the number of sugar residues in the oligosaccharide repeating units of the hexosaminoglycans. It is strongly advisable also to identify the signals belonging to the terminal carbon atoms of each sugar residue, and their number must correlate with that for anomeric ones. If the number of signals for terminal carbon atoms exceeds that for C1 atoms this indicates the presence of uncommon constituent residue, e.g. 2-aminopropanediol-1,3 (*Sh. boydii* type 8) and N-acetyl-L-serine (*E.coli* O:114). Attention should be paid to the consideration of characteristic regions for resonances of N-acetyl groups and carbon atoms linked with nitrogen. In case of *P.aeruginosa* O:3 and O:6 specific polysaccharides this led to the discovery a new class of diamino uronic acids, and in case of *Sh.boydii* type 8 and *E.coli* O:114 to the amide-type linkages between sugars and their uncommon substituents. The information from the first order analysis of the C-n.m.r. spectrum is to be correlated by the determination of sugar composition by chemical methods. For example, the physical characteristics indicate that *P.aeruginosa* O:6 type specific polysaccharide, the spectrum of which is depicted on Fig.1, was built up of four different amino sugars, among which there were two 6-deoxysugars, one uronic acid and one diamino sugar. Meanwhile, the chemical analysis led to identification of N-acetyl-D-quinovosamine and N-acetyl-D-galactosamine, whereas N-acetyl-D-fucosamine was detected only in small amounts and the fourth sugar was not found at all. From these results became evident that fucosamine was retained by acidic sugar and the latter seemed to be diamino hexuronic acid.

The advanced analysis of the C-n.m.r. spectrum should be carried out very carefully, and it is safer to proceed in parallel with chemical data when the structural features of the polysaccharide are elucidated. No doubt, the types of substitutions and the sequence of the monosaccharide residues should be determined by methylation and selective cleavage analyses, despite that necessary information of this kind is encoded in the spectrum. On the contrary, configurations of glycosidic linkages are better to be determined primarily by C-n.m.r. spectroscopy, providing that assignments of all anomeric atoms were accomplished. Indeed, the magnitude of directly bonded C1-H1 coupling constant and the value of chemical shifts for C1 atoms, as well as the resonance position for C5 and C2 (the latter in case of 2-acetamido-2-deoxyhexoses) were indicative for anomeric stereochemistry. Thus, in establishing the composition and structure of the hexosaminoglycans the C-n.m.r. spectroscopy plays a very important role. Success in the choice of this effective method of polysaccharide investigation, naturally, depends on the reliability of assignment of the

signals in the spectra, and much attention should be given to this question. The assignments must be controlled by various means, mainly by chemical modifications. For example, the unequivocal assignment of C5 atom in hexuronic acid residue is accomplished by means of alkaline titration of carboxylic groups and this causes 3 p.p.m. down-field shift in the resonance of C5 atom. Analogously, the reduction of carboxylic groups results both in appearance of new resonance of just created hydroxy methyl group and in 2 p.p.m. up-field shift of C4 atom signal of the former uronic acid residue. Useful information is provided by de-O-acetylation which affects the resonances of neighbour carbon atoms.

The great interest and challenge is, undoubtedly, the complete interpretation of the hexosaminoglycan C-n.m.r. spectrum, which, despite recent progress in this field, still remains a difficult problem. As we have found previously, this problem can be solved by using the spectral characteristics of different products derived from the original polysaccharide by means of selective cleavages, e.g. the modified polymer with no side chain and/or oligosaccharides of different length and glycosides. However, these important spectral data are, as a rule, insufficient for the complete interpretation of the spectrum. Fortunately, the spectral data for model glycosides, which are widely synthesised for n.m.r. spectroscopy purposes nearly in every carbohydrate laboratory, help to overcome this gap. The Table illustrates the signal assignments for *P.aeruginosa* O:6 specific polysaccharide after complete interpretation of its C-n.m.r. spectrum depicted at Fig. 1.

TABLE. Chemical shifts in the ^{13}C -n.m.r. spectrum of *P.aeruginosa* O:6 type specific polysaccharide.

Constituent	Chemical shifts of					
	C1	C2	C3	C4	C5	C6
-4) GalNAc (α 1-	99.7	50.6	67.8	77.4*	71.9	60.7
-4) Glc(NAc) $_2$ A (β 1-	103.1	55.6**	55.9**	73.8	78.9	
-3) FucNAc (α 1-	99.6***	49.1	78.6*	71.9	67.8	16.6
-3) QuiNAc (α 1-	99.2***	53.7	78.3*	77.9*	69.5	17.3

To achieve this interpretation, the spectral data of following compounds were used: carboxyl-reduced polysaccharide, trisaccharide 40, disaccharide 37, glycoside 38, glycoside 39, methyl 2,3-diacetamido-2,3-dideoxy- β -D-glucopyranoside, benzyl 3-O-benzyl-2-acetamido-2,6-dideoxy- β -D-galactopyranoside, benzyl 3-O-benzyl-2-acetamido-2,6-dideoxy- α -D-galactopyranoside, methyl 2-acetamido-2-deoxy- α -D-galactopyranoside, methyl 4-O-methyl-2-acetamido-2-deoxy- α -D-galactopyranoside and benzyl 3-O-benzyl-2-acetamido-2,6-dideoxy- α -D-glucopyranoside. And nevertheless, some assignments appeared to be equivocal and they are marked by asterisks. The last remark indicates that precise interpretation of the C-n.m.r. spectrum of the complex hexosaminoglycan is not yet possible. But from the other hand, the high accurateness is not of urgent necessity for the task of structural analysis in terms of primary chemical structure, and the conclusion that made-by-chemist interpretation of the spectrum is in agreement with the polysaccharide structure established by chemical methods can be considered as a reward for the efforts spent.

Selective cleavage. In spite of the significant role of physical methods, the main information on the polysaccharide structure is gained, nevertheless, by means of chemical methods, methylation analysis and selective cleavage being of decisive importance. Classical methods, i.e. Smith degradation and partial hydrolysis, were widely used in our research. However, as it became evident soon, these methods were unable to be applied to a number of hexosaminoglycans for the latter either did not include vicinal hydroxy groups or were resistant against acid hydrolysis. In the search for methods of selective cleavage of hexosaminoglycans we noted the work of Mort and Lamport (Ref. 23) on the solvolytic depolymerization of the oligosaccharide chains in glycoproteins by liquid hydrogen fluoride and applied this method to our objects. We discovered soon that, depending on the conditions, the glycosidic linkages of different types of sugars exhibited a different extent of stability under action of this reagent. The resistance towards splitting diminished in the following succession of glycosides: Hex(NAc) $_2$ A > HexNACA + HexA > HexNAc > 6-deoxy-HexN Ac + neutral sugars. The solvolysis with liquid hydrogen fluoride was successfully applied to the structural analysis of the polysaccharides from *Sh.boydii*

type 8, *E.coli* O:114 and *P.aeruginosa* O:2, O:3 and O:6, and the results obtained have been considered above. The experimental inconveniences caused by harmfulness of hydrogen fluoride and necessity to use vessels made of inert plastic are by no means considered as an obstacle to a wide use of this promising reagent.

Complicated structures. The glance at the history of polysaccharide chemistry reveals that at each stage of its development, tightly connected with the creation of a new, principal method of investigation, the belief used to arise that the polysaccharide structural analysis will be transformed into a technical task solved by means of standard and routine methods in the nearest future. This belief was especially strong (Ref.24) in the middle seventies of this century when gas chromatography combined with mass-spectrometry appeared on the scene and won the overall recognition. However, this did not happen and the impression is gradually arising that microorganisms accepted the challenge and switched their biosynthesis onto elaboration of the more sophisticated polysaccharide structures which are far from being established by conventional methods. Insuperable obstacles could be met even from the very beginning when the attempts to determine the monomeric composition fail to give a satisfactory results. For example, we have carried out the C-n.m.r. screening of the *P.aeruginosa* specific polysaccharides isolated from all known serotypes of Lanyi classification and encountered the situation when the spectra of some polysaccharides did not provide a rational information on their composition. Naturally, no recommendation could be made how to proceed in this situation and each solution seems to be an individual one.

As an example of new logic in the approach to structural analysis of the complicated polysaccharides could be considered, so-called, "retrospective logic" applied by us to the *P.aeruginosa* O:3 specific polysaccharides. The preliminary data, which were received from the spectral analysis and attempts to determine the sugar composition by analysis of the hydrolysate, have been considered in the above sections. The confusing moment was the presence in C-n.m.r. spectrum of the polysaccharide the uncommon signals at 19.8 and 167.4 p.p.m. which resembled distantly those for O-acetyl group. Not being aware of the success, we subjected the polysaccharide to mild alkaline treatment with the aim to remove this "pseudo" O-acetyl group, and discovered unexpectedly that both uncommon signals have disappeared to give the classical signals for N-acetyl group. Thus, two N-acetyl groups in one of the monosaccharide residues were somehow conjugated, and the first candidate for this derivative was imidazoline ring.

Reverting to composition of the trisaccharide repeating unit of the *P.aeruginosa* O:3 polysaccharides, we were able to think of it as composed of two inconceivable diamino acidic sugars and one N-acetyl-D-fucosamine residues. The unusual composition and the structural peculiarities of the investigated polysaccharides made it necessary to find an approach with which the new monosaccharides could be identified without being isolated in their free form i.e. directly in the course of polysaccharide structural analysis. We attempted to cleave polysaccharides along the N-acetyl-D-fucosaminidic linkages and succeeded in this after hydrogen fluoride solvolysis has been applied. Hydrogen fluoride has split the polysaccharide chain to give quantitatively the trisaccharide with N-acetyl-D-fucosamine residue on its reducing end. Comparative analysis of the C-n.m.r. spectra of the polysaccharide and the trisaccharide has shown that solvolysis did not affect the structural peculiarities of the components, and, therefore, trisaccharide obtained was the repeating unit of both polysaccharides. The approach chosen by us for structural elucidation of the trisaccharide repeating unit was aimed at a gradual simplification of the trisaccharide molecule by means of successive chemical reactions simultaneously with accumulation of the spectral characteristics for each product. The composition of the simplest disaccharide, that resulted from these modifications, became clear at last from its spectral data and was independently confirmed by the synthesis of diamino hexose which was supposed to be the constituent of the final disaccharide. From this particular moment the "retrospective logic" was put in action. The spectral characteristics of the diamino sugar isolated from the polysaccharide as a result of many successive reactions in combination with the spectral data accumulated in the course of these transformations made it possible to read retrospectively the chemical structures starting from the simplest disaccharide upto the trisaccharide and, afterwards, the polysaccharide. The method of "retrospective logic" is to be applied to the complicated polysaccharides the sugar composition of which is obscured and the only way to determine it is to establish the whole polysaccharide structure. We think that this method deserves attention as a kind of indicative vector in the tedious search for approaches to the complicated polysaccharide structures.

CONCLUSION

Bacteria *Pseudomonas aeruginosa*, *Shigella boydii* and *Shigella*-like *Escherichia coli* are shown to produce lipopolysaccharides the O-specific polysaccharide chains of which are, as a rule, acidic hexosaminoglycans. These polysaccharides are regular, linear and branched polymers composed of oligosaccharide repeating units comprising up to six monosaccharide residues. Different amino sugars and uronic acid seem to be characteristic components of these polysaccharides. As an example of the non-carbohydrate constituents, in these polysaccharides there were discovered 2-aminopropanediol-1,3 attached by amide linkage to the residue of D-galacturonic acid, and N-acetyl-L-serine also attached by amide bond to the 3-amino-3,6-dideoxy-D-glucose residue. In the specific polysaccharides from *P.aeruginosa* O:3 and O:6 there were detected and identified new sugars, which were not found in Nature earlier, i.e. 2,3-diacetamido-2,3-dideoxyhexuronic acids with D-gluco, D-manno and L-gulo configurations, as well as inconceivable 2,3-(1-acetyl-2-methyl-2-imidazolino-5,4)-2,3-dideoxy-D-mannuronic acid which was a kind of chimeric fusion of pyranose and imidazoline rings.

These new diamino uronic acids were a significant obstacle for the polysaccharide structural analysis. The difficulties were overcome by the intensive use of physical methods tightly combined with chemical routine and newly developed approaches. The acidic amino sugars prevented the complete acid hydrolysis of the hexosaminoglycans, and thus distorted the results of quantitative sugar analysis causing the erroneous chemotyping of antigens. The accomplished investigation is expected to stimulate further research on the biosynthesis of the newly discovered sugars and the pathways of their transfer into the growing polysaccharide chains. No doubt, the structures established are a challenge for synthetic chemistry because their synthesis will require the development of new methods. The synthesised diamino sugars and their oligosaccharides will be of great interest for microbial immunology as immunodeterminants for constructing artificial antigens imitating the immunological specificities of the native antigens, as well as the whole microbial cells. And, at last, we believe that the chemical structures of the O-specific polysaccharides will serve as objective molecular criteria in eliminating numerous contradictions in the existing serological classifications of *P.aeruginosa* species.

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