SYNTHESIS OF FRAGMENTS OF BACTERIAL POLYSACCHARIDES AND THEIR APPLICATION FOR THE PREPARATION OF SYNTHETIC ANTIGENS

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Abstract — Different approaches towards preparation of Salmonella O-specific polysaccharides are compared. They include stepwise chain elongation using synthons derived from oligosaccharide repeating units, chemical polycondensation of protected 1,2-O-cyanoethylidene-trisaccharides containing O-trityl group, and enzymatic polymerization of repeating units in polyacryl pyrophosphate oligosaccharides prepared by chemical synthesis (chemical-enzymatic approach). Synthesis of artificial antigens through co-polymerization of acrylamide and allyl glycosides of oligosaccharides is described. Oligosaccharide fragments of Salmonella O-specific polysaccharides and Str. pneumoniae type 3 capsular polysaccharide were used for preparation of these antigens.

INTRODUCTION

Development of theoretical concepts put forward by immunochemists as well as the advances in applied immunology made the question of creation of synthetic antigens possessing definite specificity practicable. On the other hand, progress in carbohydrate chemistry allows to elucidate, fast and conclusively, the structure of somatic antigens of Gram-negative bacteria and, particularly, of their specific polysaccharide chains. Currently, the structure of more than 300 of such polysaccharides was established. All these compounds, which play a role of the specific hapten within the antigenic complex, possess a strictly regular structure and represent block-polymers made up of repeating units (Ref. 1). It is known that immunochemical determinants which are responsible for serological specificity can be found within one such unit, or in combination of two adjacent units.

The most significant progress is achieved in the chemistry of somatic antigens of Gram-negative bacteria, that is why it is in this field that the most significant investigation on the synthesis of artificial antigens were initiated. I should like to report what has been performed recently in this direction in our laboratory.

Basically, a synthetic antigen can be developed for several purposes: i) to produce reagents of narrow specificity for a serological analysis, and to prepare the diagnostic sera; ii) for the use as a basis of the specific preventive vaccines (in this case an antigen must be also immunogenic and
the higher antibody titers); iii) for the utilisation as a medical preparation (in this case it must afford the protective effect and meet the stringent clinical demands). Distinct destination of the antigen raises different requirements to the structure of the synthetic antigen.

Preparation of a synthetic antigen involves: i) synthesis of a corresponding hapten bearing either narrow or wider specificity and ii) addition of a hapten to a polymeric matrix to endow it with necessary physico-chemical properties and proper immunogenicity. Generally, fragments of carbohydrate chains bearing the corresponding determinant, the so-called O-factor (Ref. 2) or a whole repeating unit itself, are employed as haptens. In the case when wider specificity of an antigen is needed, more complicated combination of the hapten fragments is apparently required. Synthesis of oligosaccharides which are such specific fragments is presently one of the hot points in the development of carbohydrate chemistry. Synthesis of the complete repeating units of the O-antigenic polysaccharides of Gram-negative bacteria, exemplified by Salmonella, was initiated in our laboratory about 10 years ago (Ref. 3). Presently, syntheses of such a kind are carried out in many other laboratories and are extensively documented in the literature. In this connection, the first part of my lecture will be devoted to the realization of the next step, namely, the preparation of the total hapten of Gram-negative bacteria through the assembling of such repeating units to prepare the polymeric structures corresponding to the natural hapten.

Three different pathways to achieve this aim are conceivable (cf. Ref. 4): i) sequential addition of oligosaccharide repeating units (stepwise synthesis), ii) polymerization of oligosaccharide repeating units by chemical methods, and iii) enzymatic polymerization of oligosaccharide repeating units. It is quite obvious that to obtain the compound totally corresponding to either natural biopolymer or its fragment, the joining of oligomeric units must be performed with strict regio- and stereospecificity. Apparently, even a single faux pas in the formation of new glycosidic linkages may result in a distortion of the polymeric structure and conformation, and, consequently, in alteration of its physico-chemical and biological properties. Thus, the glycosylation reactions, which form the basis for the unit joining, subject to severe requirements. The most serious problem is to maintain the absolute stereospecificity of the glycosidic linkage formation. Our laboratory had accumulated certain experience in all three directions quoted above.

**STEPWISE SYNTHESIS**

This pathway seems to be the most obvious, while, at the same time, it is the most laborious one. In order to convert the process of extension of a polymeric chain into a standard procedure it is necessary to functionalize the oligosaccharide repeating unit of a bacterial polysaccharide in such a manner as to generate the aglycon and glycosylating synthons. Generally, it is functionalization of "monomer" which is the most laborious step. Let me exemplify the realization of such an approach by sequential addition of repeating units of the O-antigenic polysaccharide of *Salmonella newington* (Ref. 5). The latter
polysaccharide is known (Ref. 6) to consist of the repeating units (1) with \(\beta-(1\rightarrow 6)\)-linkage between Gal and Man.

\[ \beta-D-Manp(1\rightarrow 4)-\alpha-L-Rhap(1\rightarrow 3)-D-Gal \quad (1) \]

The synthesis of a "dimer" of such a repeating unit was carried as shown in Fig. 1. We had proposed several schemes for the synthesis of the trisaccharide (1), the last of which (Ref. 7) allows to prepare it in a most efficient way.

The first stage in the synthesis of the "dimer" was the preparation of the derivative (2), which served as a starting compound for both the aglycon (7) and glycosylating (4) synthons. To prepare (4), the compound (2) was converted into decaacetate (3), and then into glycosyl halide (4), its purity is of critical importance for the success of the subsequent condensation; this stage ought to be thoroughly controlled. Preparation of (7) concluded deacetylation of (2), the only primary hydroxyl group in (5) was methoxytritylated, the resulting (6) was then successively benzylated and detritylated to afford the aglycon synthon (7). Helferich condensation of (4) and (7) was carried out using high-vacuum technique to avoid moisture completely (cf. Ref. 8). The yield of the hexasaccharide derivative (8) after chromatography amounted to 80%, the formation of \(\alpha\)-anomer being quite negligible. Removal of protecting groups gave the free hexasaccharide, a "dimer" of the repeating unit.
To make the next stage in the stepwise synthesis the "dimer" (9) was converted into peracetate and then into glycosyl halide (10), which then reacted with (7) to give the nonasaccharide derivative (yield 57%) whose deprotection gave a "trimer" of the repeating unit (11) (Fig. 2).

\[
\begin{align*}
9 & : R = H; \quad X = OH \\
10 & : R = Ac; \quad X = Br \\
\beta - \text{Man} \ (1-4) - \alpha - \text{Rha} (1-3) - \beta - \text{Gal} (1-6) - \beta - \text{Man} (1-4) - \alpha - \text{Rha} (1-3) - \beta - \text{Man} (1-4) - \alpha - \text{Rha} (1-3) - \text{Gal}
\end{align*}
\]

Fig. 2.

Such a stepwise extension of a polymeric chain can be carried on using the same sequence of reactions and the standard aglycon (7). It should be kept in mind, however, that the yields of condensation products would probably decrease with increasing chain length, while removal of the \( \alpha \)-anomeric impurity would become more and more difficult. This route to prepare higher oligosaccharides is certainly very laborious, while it allows to obtain pure oligomer of definite structure with given degree of unit recurrency.

POLYMERIZATION BY CHEMICAL METHODS

This most straightforward approach to obtain polymer of strictly regular structure was quite inaccessible until recently, since it necessitates the glycosylation reactions to be carried out with absolute stereospecificity. It is quite obvious that the polycondensation reaction, yielding even a small amount of "spurious" units, would result in a sterically irregular polymer. Repeated attempts to obtain polymers using various versions of the Koenigs-Knorr and orthoester glycosylation reactions made us sure of the fact. The realization of this approach became possible only when a new glycosylation reaction, based on interaction of trityl ethers with 1,2-0-cyanoethyldiene derivatives of sugars, which proceeds in the most cases studied with absolute stereospecificity and results in 1,2-trans-glycosides (Ref. 8), was discovered in our laboratory (Fig. 3). This reaction proceeds in the presence of 10 mol.% of triphenylmethylum perchlorate and represents a heterolytic catalytic process, which seems to occur according to a concerted mechanism, thus providing absolute
A general method was developed on the basis of this reaction for the synthesis of both homo- and heteropolysaccharides, repeating units in which are linked by 1,2-trans-glycosidic bonds (for reviews see Ref. 9, 10). Monomers for the polycondensation were the derivatives of mono- and oligosaccharides, which bear the 1,2-0-cyanoethylidene moiety at the reducing end, and the O-trityl group at a site where new glycosidic bond is to be formed (Fig. 4).

This method proved to be applicable for a rather wide range of sugars, including derivatives of uronic acids and aminosugars, although we are already aware of some exceptions where stereospecificity of glycosylation is lost and degree of polymerization is low.

We exemplify this method by the synthesis of the O-antigenic polysaccharide of Salmonella newington (Ref. 11). In this case, the properly functionalized trisaccharide (1) was subjected to polycondensation. Synthesis of the "monomer" for polycondensation, which contains the trityl group at 0-6 of the mannose residue and cyanoethylidene group at the reducing end, is presented in Fig. 5.
The peracetate (3) was converted into glycosyl halide and then into the 1,2-O-\cynoethylidene derivative (12), which was deacetylated by dilute sodium methoxide and then tritylated. This last, most critical, step of the synthesis afforded a mixture of two mono- and one ditrityl derivatives. Fortunately, it was possible to separate these derivatives by chromatography after acetylation to give a monomer (13). Its polycondensation was performed under the standard conditions (10 mol.% of catalyst, 20°C, dichloromethane, vacuum technique to avoid moisture), the process practically came to an end after 50-60 h and was finally terminated by addition of aqueous trifluoroacetic acid. The polymer obtained was deacetylated, and the free polysaccharide (14) was subjected to fractionation on BioGel. Naturally, the polysaccharide obtained was a mixture of polymer-homologues. A fraction with mol. mass of ca. 5000 was isolated for further studies (yield 60%). This corresponds to a degree of polymerization of 11-12 and coincides with an average mol. weight of the natural O-antigenic polysaccharide of Salmonella newington (Ref. 12). The structure of (14) was confirmed by acid hydrolysis and methylation analysis. Configuration of the newly formed glycosidic linkages between galactose and mannose residues was subjected to the most thorough control. The synthetic polysaccharide was subjected to Smith degradation, which led to oxidation of the mannose and rhamnose residues leaving intact the galactose residue, and resulted in 1-O-\galactopyranosyl-glycerol (15).

\[
13 \rightarrow \beta-\text{Man(1-4)}-\alpha-\text{Rha(1-3)}-\beta-\text{Gal(1-6)}-\beta-\text{Man(1-4)}-\alpha-\text{Rha(1-3)}-\beta-\text{Gal...}
\]

![Fig. 6.](image)

Determination of anomeric configuration of the galactose in this glycoside allows to establish unambiguously the configuration of the newly formed galactose-mannose bond. Thorough analysis of (15) using capillary GLC and the authentic \(\alpha\)- and \(\beta\)-isomers of (15) proved the degradation product to be the pure \(\beta\)-isomer (with an accuracy of 1%). This confirms conclusively the total stereoregularity of (14) and absolute specificity of the polycondensation reaction for the monomer (13). The \(^{13}\text{C}\) NMR spectra of the synthetic polysaccharide (14) and the polysaccharide isolated from S. newington also showed a complete identity over all eighteen carbon atoms.
Still more essential results were observed on the immunochemical testing of the synthetic polysaccharide (14), which showed a strong inhibitory activity in the passive haemagglutination reaction with 0-3-anti-3-serum from Salmonella. However, corroboration of high specificity of the synthetic hapten (14) would be, perhaps, of particular interest. To this aim we have synthesized its closest analogue, the polysaccharide (16) with the α-configuration of the mannosyl-rhamnosyl linkages (Ref. 11). The latter proved to be practically inactive in the reaction with the same serum.

\[ \text{\ldots } \alpha-\text{Man} (1-4)-\alpha-\text{Rha}(1-3)-\beta-\text{Gal}(1-6)-\alpha-\text{Man}(1-4)-\alpha-\text{Rha}(1-3)-\beta-\text{Gal} \ldots \]

The data obtained evidence conclusively in favour of the synthetic hapten (14) being not only active, but exhibiting high specificity, and its identity to the natural O-antigenic polysaccharide is beyond any doubt. The example presented is the first synthesis of the complex, natural, biologically specific heteropolysaccharide by purely chemical methods. It is evident that this method can find wider application. The most complicated part of the work is the synthesis of the properly functionalized oligosaccharide monomer.

The method of chemical polymerization is the most direct, although rather complex way to prepare polymeric haptens. In its present state, the method is restricted by its failure to give polysaccharides with the 1,2-cis-linkage, as well as those possessing the 1→2-glycosidic linkage between the repeating units.

ENZYMATIC POLYMERIZATION (CHEMICAL-ENZYMATIC APPROACH)

Limitations of the aforementioned approaches and, in particular, their failure to prepare polysaccharides with 1,2-cis-glycosidic linkages between repeating units made us to look for other pathways. Biosynthesis of bacterial polysaccharides of such a type is known (Ref. 13) to proceed by assembling the repeating unit on a lipid carrier (higher polyprenol) followed by polymerization of this unit under action of membrane-bound polymerase. It is clear that a synthetic precursor - polyprenyl pyrophosphate oligosaccharide (20), whose oligosaccharide chain corresponds to the repeating unit - can be polymerized in a similar way. It is understood that this approach can be considered reasonable from the preparative point of view if it exhibits sufficient universality and synthesis of the starting material is not too laborious and specific. Two factors make this approach quite practical. First, it was established (Ref. 14) that specificity of bacterial polymerases is not completely strict. This significant discovery allows to use the polymerase of a given bacterium to synthesize not only its own polysaccharide but also to polymerize the precursors with the modified oligosaccharide units, including synthetic derivatives as well. Second, it turned out that the practically unavailable bacterial polyprenol can be effectively substituted by the readily available plant polypre-
nol (Ref. 15) in the synthesis of a precursor. For this purpose we make use of a thoroughly studied polyprenol isolated from mulberry leaves (Ref. 16). Taking into account these factors, the chemical-enzymatic method provides sufficiently wide applicability and may be of considerable preparative value.

We exemplify the latter approach by the synthesis of the O-antigenic polysaccharide of Salmonella anatum (21), consisting of the same repeating units, mannose-rhamnose-galactose, joined by α-galactosidic, i.e., 1,2-cis-linkage, which is inaccessible by the chemical polymerization method (Fig. 7).

For convenient assay of the polymerization reaction a tritium label was incorporated into the trisaccharide (1) at C-6 of the galactose residue (Ref. 17). The labelled trisaccharide (17) was converted into α-1-phosphate (18). In this case, phosphorylation of the peracetate of (17) with anhydrous phosphoric acid proved to be the most successful method, since it provided for minimal scission of glycosidic linkages (Ref. 13). Polyprenol from mulberry leaves, moraprenol, was converted into phosphate by treatment with O-phenylene phosphochloridate (Ref. 16), and further into respective imidazolidate (19). Condensation of the phosphates (18) and (19) gave a pyrophosphate (20), a precursor for the enzymatic polymerization (Ref. 19). At first synthesis of (20) faced serious difficulties, since in the only reported (Ref. 20) procedure for the preparation of polyprenyl pyrophosphate sugars acetates of phosphorylated sugars were used as starting materials, while deacetylation of the pyrophosphates obtained was accompanied with practically total rupture of the pyrophosphate linkage. In this connection, a new procedure was developed to prepare pyrophosphates through phosphoimidazolides (Ref. 19), which allowed to introduce the phosphates of unprotected oligosaccharides into pyro-
phosphate synthesis. This method of synthesis of polyprenyl pyrophosphate oligosaccharides appears to be sufficiently general, and it thus opens the route to the synthesis of polysaccharides of various structure.

It is known that polymerases of the bacterial cell enter the enzymatic ensemble providing for biosynthesis of the O-antigenic polysaccharides, and are strongly attached to the membrane. All attempts to solubilize and purify these enzymes failed as yet. That is why at present one has to employ a membrane preparation containing the corresponding polymerase. In our case a preparation from cell envelope of Salmonella anatum was employed. Incubation of (20) with this preparation, followed by mild acid hydrolysis to split off the terminal polyprenyl pyrophosphate residue, afforded the polysaccharide (21), which was then purified by gel-filtration. A fraction with an average polymerization degree of ca. 5 (mol. weight 2500-3000) was isolated in 80% yield. The structure of (21) was confirmed by methods similar to those described above, involving isolation of 1-O-(α-galactosyl)-glycerol after Smith degradation, as well as enzymatic hydrolysis with α-galactosidase from coffee beans.

It was already pointed out that polymerases possess rather wide specificity. As a result the structure of the oligosaccharide fragment can be altered within the polymerization precursor (20) due to a wide specificity of the polymerase. Thus, according to the preliminary data (Ref. 14, 21), the D-galactose residue can be substituted by D-fucose, D-glucose, D-talose, or 4-deoxy-D-xylohexose, while the D-mannose residue - by either D-rhamnose, 3-deoxy-D-arabino-hexose or D-glucose. All these modified precursors can also be polymerized by polymerases from Salmonella anatum, S. senftenberg or S. typhimurium, the reaction, however, proceeding at slower rates and with lower yields.

Although the preparative aspects and limits of the chemical-enzymatic approach have still to be elaborated, there is no doubt of its wide-spread applicability. Provided some difficulties are overcome, in particular, an access to standard polymerase preparations of higher degree of purity, the chemical-enzymatic method may become an indispensable instrument for the synthesis of many bacterial polysaccharides containing also 1,2-cis-glycosidic inter-unit linkages.

Different synthetic approaches to prepare haptens of antigens of Gram-negative bacteria prove to offer wide opportunities and allow to solve many complicated problems of bacterial immunochemistry.

SYNTHETIC ANTIGENS

The second part of my report deals with the question of a polymeric carrier, to which haptens are attached to endow synthetic antigen with a proper molecular weight and immunogenicity. In the classical works by Goebel (22) the problem was solved by attaching the haptenic units to a protein. This method, brought later to perfection (Ref. 23), becomes presently of common use and is widely employed for production of antigenic preparations. One of the serious
drawbacks of this approach is the incorporation into a synthetic antigen of a protein chain, which per se displays antigenic properties in many cases and, thereby, causes formation of antibodies of quite different specificity than the hapten attached to this chain. In addition, due to presence of protein moiety and branched-chain architecture, such antigens are T-dependent, as opposed to T-independent natural carbohydrate antigens (Ref. 24). Finally, since the attachment of the hapten by existing methods proceeds mainly through the ε-aminogroups of the lysine residues, the "concentration" of haptenic group in the synthetic antigen turns out to be restricted by the number of this aminoacid residues.

These difficulties can be overcome if it becomes possible to substitute the protein polymeric matrix by another polymer. We have chosen polyacrylamide as such a polymeric carrier, bearing in mind that its polymeric chain resembles, to some extent, polypeptide chain, while this macromolecule can be readily modified by means of introduction of cross-linkages. The polymer with a polyacrylamide backbone can be prepared by copolymerization of acrylamide with a hapten, bearing suitable group, as it was demonstrated by Kocourek in lectin research (25).

As the object for our study we have chosen several synthetic antigens, containing i) total repeating unit, namely, trisaccharide (1), which is characteristic of Salmonella anatum and S. newington; ii) monofactor determinant of several types of Salmonella, namely, of groups B (factor 04) and D (factor 019); iii) three oligosaccharides, which are close in structure to the repeating unit of one of the simplest capsular polysaccharides Streptococcus pneumoniae type 3. Let me demonstrate the preparation of the antigen, involving the repeating unit of S. anatum as a hapten (Ref. 26). Allyl β-glycoside of the trisaccharide (1) was prepared for the copolymerization with acrylamide. Synthesis of the former is presented in Fig. 8. Allyl β-galactoside (22) was subjected to isopropylideneation, acetylation, and mild hydrolysis to give 2,6-diacetate of allyl β-galactoside (23); the latter was treated with triethyl ortho-acetate in the presence of p-toluenesulphonic acid, the resulting ortho-ester was hydrolysed with 80% acetic acid to give 2,4,6-triacetate of allyl β-galactoside (24). Condensation of (24) with acetobiosyl bromide (25), generated from the corresponding mannosyl-rhamnose, produced peracetate (26) in moderate yield, from which allyl glycoside (27) of the trisaccharide (1) was isolated by Zemplen deacetylation. To prepare antigen, the glycoside (27) was subjected to radical copolymerization with acrylamide (the ratio of monomers is 2 : 1) in the presence of ammonium persulfate and N,N,N',N'-tetramethylethylenediamine. The resulting copolymer (28) was isolated by gel-filtration on Sephadex G-50; the copolymer contained ca. 30% of carbohydrates, i.e., the ratio of the acrylamide and hapten units is 13 : 1; its molecular weight, determined by ultrafiltration and sedimentation analysis, amounted to 100,000-300,000. The structure of the copolymer was confirmed by sugar analyses and 13C NMR spectrum which contained all the necessary signals. Thus, there was no alteration of the carbohydrate hapten during the copolymerization. Changing the ratio of the copolymerization components allows to vary the number of hap-
Antigenic properties of \( (28) \) were studied in passive haemagglutination inhibition and by double immunodiffusion according to Ouchterlony with the homologous and heterologous sera. The synthetic antigen \( (28) \) was shown to possess a monofactor 0:3 specificity of Salmonella (Ref. 27), being even superior to the natural lipopolysaccharide in efficiency of the serological reaction (100 times as high according to the Ouchterlony test, 10 times as high according to the inhibition test). The latter fact is apparently related to a higher "concentration" of haptons in the synthetic antigen, since decreasing the "concentration" of the determinant down to 2% results in the serological activity of the synthetic antigen matching that of the natural lipopolysaccharide (LPS). Further, \( (28) \) possessed the immunogenic properties with no toxicity, causing the animals (mice and rabbits) to elicit antibodies with narrow specificity against the 0:3 factor without any need in adjuvant (Ref. 27).

The immunogenicity of \( (28) \) is comparable to that of LPS, and after the three-fold immunization of mice with both the synthetic antigen and LPS, the antibody titre in sera is roughly equal and rather high. Finally, mice immunized with the copolymer \( (28) \) acquire anti-endotoxic and anti-bacterial immunity, providing for survival of ca. 70% of mice, given with a lethal dose of LPS or the living culture (Ref. 28). These results indicate clearly that the synthetic antigens, devoid of protein matrix, display not only high serological activity, but also manifest high immunogenicity. Still more interesting is the occurrence of protectivity, although this property has yet to be thoroughly studied. Thus, this new principle of creating the synthetic antigens, featuring simplicity and convenience for a large-scale production, deserves, from our
viewpoint, particular attention.

It is noteworthy that the architecture per se of a synthetic antigen differs from that of the natural one, since the haptenic groups in (28) are arranged as side chains, whereas in the natural O-antigenic polysaccharide these are incorporated into the main chain. In this connection a question arises as to what extent the specificity of the synthetic and natural antigens is adequate. For this purpose the synthetic antigens were prepared, which contained haptens with the group factors 0:4 (abequosyl-mannose hapten), 0:9 (tyvelosyl-mannose hapten), and 0:2 (paratosyl-mannose hapten), and the degree of their specificity was tested. Synthesis of the corresponding haptens was performed following the schemes, which are close to those presented by Swedish group, with modification allowing to prepare the necessary haptens as allyl glycosides. Synthesis of one of them (Ref. 26), namely, allyl 3-O-(abequosyl)-D-mannopyranoside, is shown in Fig. 9. Allyl 2,3:4,6-di-O-isopropylidene-α-D-mannopyranoside (29), prepared by acetonation of allyl-α-D-mannopyranoside, was subjected to mild acid hydrolysis in aqueous acetone. The resulting 2,3-O-isopropylidene derivative (30) was acetylated, de-isopropylidened, and the 4,6-diacetate (31) through 2,3-orthoacetate, was converted into 2,4,6-triacetate of allyl-α-D-mannopyranoside (32), which was subjected to Helferich glycosylation by 2,4-di-O-(p-nitroterzoyl)-α-D-abequosyl bromide to give the disaccharide derivative (33), which, when treated with barium oxide in boiling methanol, afforded allyl α-glycoside of abequosyl-mannose (34).

![Chemical structures](image)

The hapten (34) was copolymerized with acrylamide (in ratio of 2:1) under conditions described above; the copolymer (35) (Fig. 10) of molecular weight 100,000-300,000 was obtained in 25% yield; it contained ca. 30% of carbohydrates, that is the ratio between (34) and acrylamide units was equal to 1:10. In a similar way the copolymer was prepared with disaccharide tyvelosyl-mannose (36), and paratosyl-mannose-containing polymer (37) is now in preparation. Biological testing of the synthetic antigens (35) and (36) were performed using both inhibition of the passive haemagglutination reaction and ELISA-technique. In the reaction with the corresponding monospecific antisera these
antigens showed strict specificity with no cross-reactions. At the same time, lipopolysaccharide obtained from a bacterium shows generally the cross-reactions with heterologous antisera, thus demonstrating the occurrence of several O-specificities inherent to that LPS. It should be emphasized that in this case activity of artificial antigens is also by 1-2 orders of magnitude as high as that of the native LPS. These experiments demonstrate that protein-free antigens of the type considered may serve as a perfect basis for the creation of diagnostic preparations. In fact, the antigens obtained were already employed successfully for the identification of some cases of salmonelloses in clinics.

\[
\begin{align*}
\text{CONH}_2 & \quad \text{CONH}_2 \\
(\text{CH}_2\text{CH})_x & \quad (\text{CH}_2\text{CH})_y \\
\text{CH}_2\text{OR} &
\end{align*}
\]

Fig. 10.

The proposed principle for the creation of synthetic antigens had been successfully applied for the preparation of the antigens of a capsular polysaccharide. One of the simplest examples of such a type was selected, namely, the antigen of Str. pneumoniae type 3, which was known to produce the capsular polysaccharide (38), consisting of the cellobiouronic acid repeating units.

\[\text{38} \quad -3\beta\text{-GlcA}(1-4)-\beta\text{-Glc}(1-3)-\beta\text{-GlcA}(1-4)-\beta\text{-Glc}(1-3)\]

At the same time, the antigenic determinant of this polysaccharide is not strictly known, thus, to prepare the optimum synthetic antigen we had also to solve another problem, namely, to determine the antigenic site of Str. pneumoniae type 3. To this effect, we have synthesized the copolymer involving three different haptens. The synthesis of the copolymer containing cellobiouronic acid residues is presented in Fig. 11. Allyl \(\beta\)-cellobioside (39) was converted into 4',6'-O-benzylidene derivative (40) and pentaacetate (41). Sequential debenzylidenation, tritylation, acetylation, and detritylation yield 2,3,4,2',3',4'-hexaacetate of allyl \(\beta\)-cellobioside (42). Oxidation of the latter by the Jones reagent followed by deacetylation, afforded allyl \(\beta\)-glycoside of cellobiouronic acid (43), which is the haptenic component of the copolymer. Copolymerization of (43) with acrylamide was carried out under standard conditions described above. The polymer obtained (44) had the molecular weight of 100,000 - 300,000. At the (43), acrylamide ratios equal to 2:1, 1:1, and 1:2 the polymers were obtained which contained 27, 13, and 3% of carbohydrate determinants, respectively.
The synthesis of the haptenic component for the synthetic antigen containing pseudoaldobiouronic acid residues, is shown in the Fig. 12. Methyl 5-O-acetyl-1,2-O-isopropylidene-α-D-glucofuranuronate (45), prepared by known methods from D-glucuronolactone, was glycosylated by acetobromoglucose under Helferich
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conditions, \( \beta \)-anomer of the pseudoaldobiouronic acid derivative (46) was deprotected and converted via acetylbiosyl halide into allyl glycoside (47), the methyl ester was hydrolyzed by 2% KOH in aqueous alcohol at room temperature to give the haptenic component (48) for the synthetic antigen. Copolymerization of (48) with acrylamide afforded the copolymer (49) which is closely related to the previous one: molecular weight is 100,000 - 300,000, carbohydrate content ca. 30%, hapten : acrylamide ratio is 1:10. Presently, the synthesis is close to completion of the haptenic component bearing the residue (50).

\[
\begin{align*}
\beta-\text{GlcA(1-4)}-\beta-\text{Glc(1-3)}-\beta-\text{GlcA} \\
(50)
\end{align*}
\]

The synthesis consists in glycosylation of the aglycon synthon (45) by glycosyl bromide derived from cellobiouronic acid, followed by conversion of the resulting trisaccharide into allyl \( \beta \)-glycoside and copolymerization of the latter with acrylamide.

Serological specificity of the prepared synthetic antigens was tested by the ELISA technique for the copolymer with cellobiouronic acid residue as a hapten. The copolymers containing 27 and 13% of carbohydrates exhibited distinct reaction with antisera against Str. pneumoniae of the serotype 3. They exhibited weak, if at all, reactivity towards antisera against 15 other serotypes. The copolymer containing 3% of carbohydrates did not practically react in all cases. Thus, the data presented indicate high specificity of this synthetic antigen. Consequently, the new principle for the preparation of antigens, outlined in this report, appears to be quite general, this is also suitable for the preparation of capsular carbohydrate antigens.

REFERENCES