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-trisaccha-
rides containing O-trityl group, and enzymatic polymerization of re-
peating units in polyprenyl 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, prog-
ress 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 haptens within the antigenic complex, possess a strict-
ly 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 combina-
tion 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 sig-
nificant 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 pre-
pare the diagnostic sera; ii) for the use as a basis of the specific preven-
tive vaccines (in this case an antigen must be also immunogenic and    elicit

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 cli-
nical demands). Distinct destination of the antigen raises different require-
ments 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 pro-
perties 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 hap-
ten 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 <u>Salmonel-
la</u>, 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 assemb-
ling 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 <u>faux</u> <u>pas</u>
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 gly-
cosidic 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 oligo-
saccharide repeating unit of a bacterial polysaccharide in such a manner as to
generate the aglycon and glycosylating synthons. Generally, it is functionali-
zation 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 <u>Salmonella</u> <u>newington</u> (Ref. 5). The latter

polysaccharide is known (Ref. 6) to consist of the repeating units (1) with
$\beta$-(1-6)-linkage between Gal and Man.

$\beta$-D-Manp(1→4)-$\alpha$-L-Rhap(1→3)-D-Gal      (1)
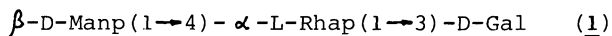
The synthesis of a "dimer" of such a repeating unit was carried as shown in
Fig. 1. We had proposed several schemes for the synthesis of the trisaccharide
(1), the last of which (Ref. 7) allows to prepare it in a most efficient way.



3. R= Ac; X= OAc        5. R=R'= H
4. R= Ac; X= Br         6. R= H; R'= p-MeOTr
                        7. R= CH$_2$Ph; R'= H
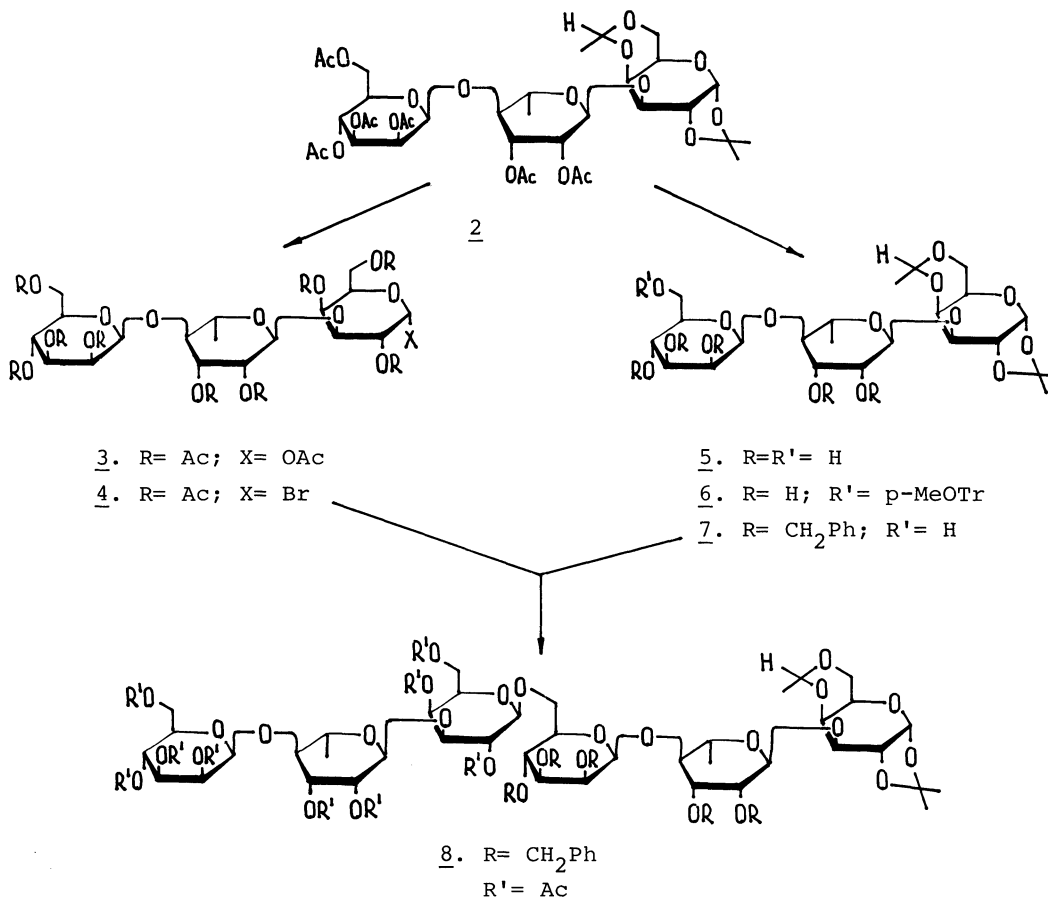
8. R= CH$_2$Ph
   R'= Ac

Fig. 1.

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 cri-
tical 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).



9.  R= H; X= OH
10. R= Ac; X= Br

+ 7
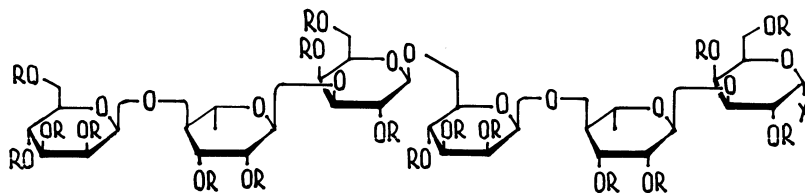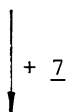
$\beta$-Man (1-4)-$\alpha$-Rha(1-3)-$\beta$-Gal(1-6)-$\beta$-Man(1-4)-$\alpha$-Rha(1-3)-$\beta$-Gal(1-6)-$\beta$-
  -Man(1-4)-$\alpha$-Rha(1-3)-Gal

11

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 decrea-
se with increasing chain length, while removal of the $\alpha$-anomeric impurity
would become more and more difficult. This route to prepare higher oligosaccha-
rides 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 struc-
ture was quite inaccessible until recently, since it necessitates the glycosyl-
ationreactions 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
attemptsto 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-O-cyanoethylidene derivatives of su-
gars, which proceeds in the most cases studied with absolute stereospecificity
and results in 1,2-trans-glycosides (Ref. 8), was discovered in our laborato-
ry (Fig. 3).This reaction proceeds in the presence of 10 mol.% of triphenyl-
methylium perchlorate and represents a heterolytic catalytic process, which
seems to occur according to a concerted mechanism, thus providing absolute
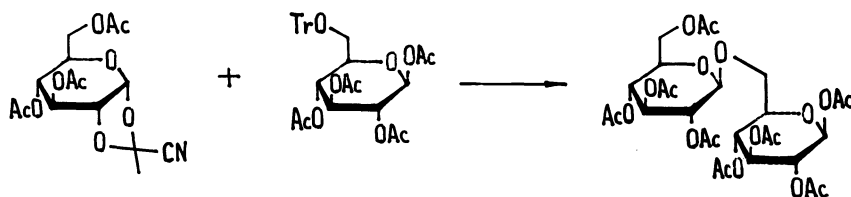
stereospecificity.

Fig. 3.

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-O-cyanoethylidene moiety at the reducing end, and the O-trityl group at a site where new glycosidic bond is to be formed (Fig. 4).
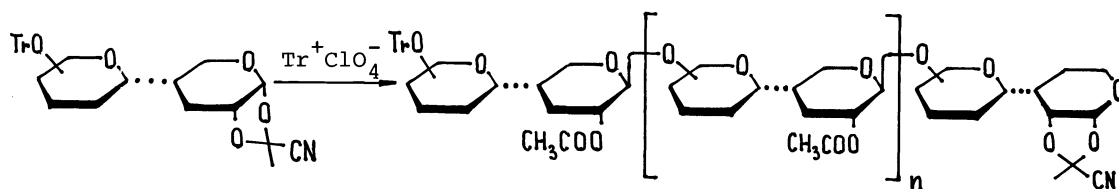
Fig. 4

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 O-6 of the mannose residue and cyanoethylidene group at the reducing end, is presented in Fig. 5.
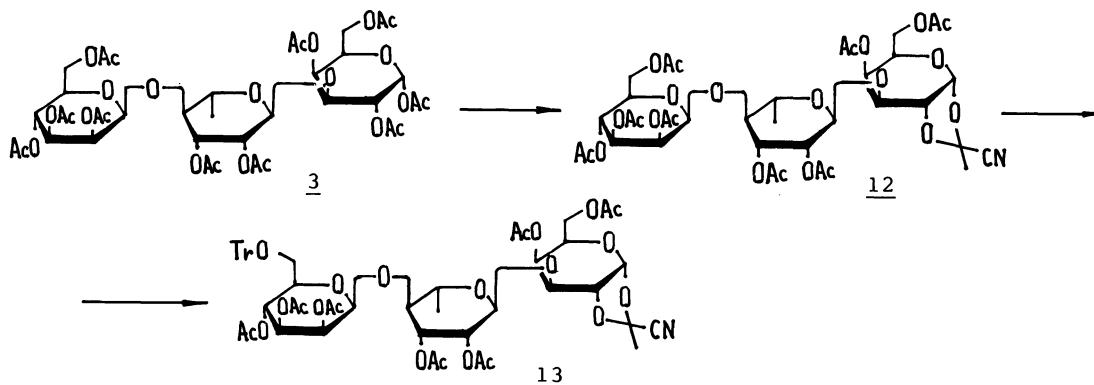
Fig. 5.

The peracetate (3) was converted into glycosyl halide and then into the 1,2-O-
-cyanoethylidene derivative (12), which was deacetylated by dilute sodium me-
thoxide 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, $20^{\circ}C$, 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 ⟶ ... $\beta$-Man(1-4)-$\alpha$-Rha(1-3)-$\beta$-Gal(1-6)-$\beta$-Man(1-4)-$\alpha$-Rha(1-3)-$\beta$-Gal...

$$\begin{array}{l} HIO_4 \\ NaBH_4 \\ H^+ \end{array}$$          14
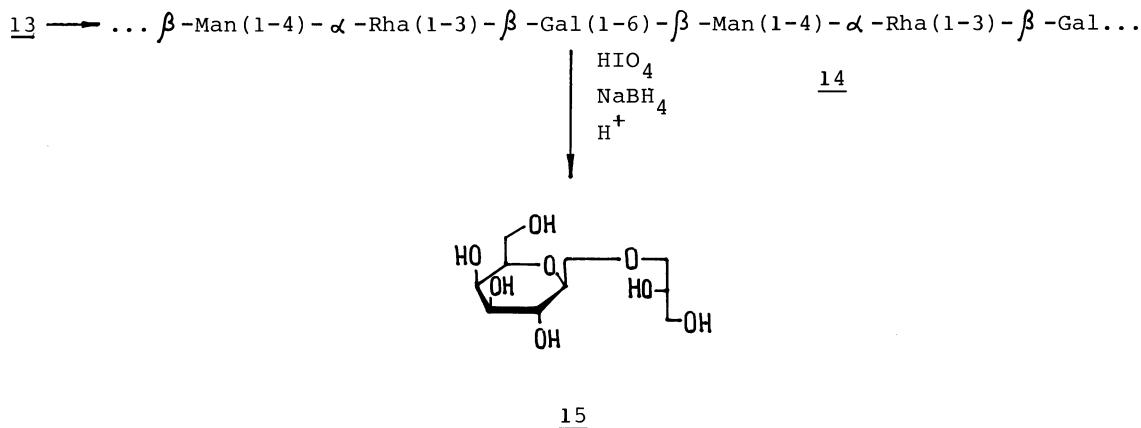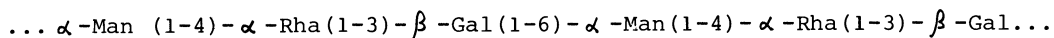


15

Fig. 6.

Determination of anomeric configuration of the galactose in this glycoside
allows to establish unambiguously the configuration of the newly formed galac-
tose-mannose bond. Thorough analysis of (15) using capillary GLC and the authen-
tic $\alpha$- 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 ste-
reoregularity of (14) and absolute specificity of the polycondensation reacti-
on for the monomer (13). The $^{13}$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 Salmonel-
la. 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 $\alpha$-configuration of the man-
nosyl-rhamnosyl linkages (Ref. 11). The latter proved to be practically inac-
tive in the reaction with the same serum.

$$\ldots \alpha\text{-Man }(1\text{-}4)\text{-}\alpha\text{-Rha}(1\text{-}3)\text{-}\beta\text{-Gal}(1\text{-}6)\text{-}\alpha\text{-Man}(1\text{-}4)\text{-}\alpha\text{-Rha}(1\text{-}3)\text{-}\beta\text{-Gal}\ldots$$
$$\underline{16}$$

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 presen-
ted is the first synthesis of the complex, natural, biologically specific he-
teropolysaccharide by purely chemical methods. It is evident that this method
can find wider application. The most complicated part of the work is the syn-
thesis 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 polysaccha-
rides 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 oligosacchari-
de 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 fac-
tors make this approach quite practical. First, it was established (Ref. 14)
that specificity of bacterial polymerases is not completely strict. This signi-
ficant discovery allows to use the polymerase of a given bacterium to synthe-
size 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 poly-
prenol 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 suf-
ficiently wide applicability and may be of considerable preparative value.

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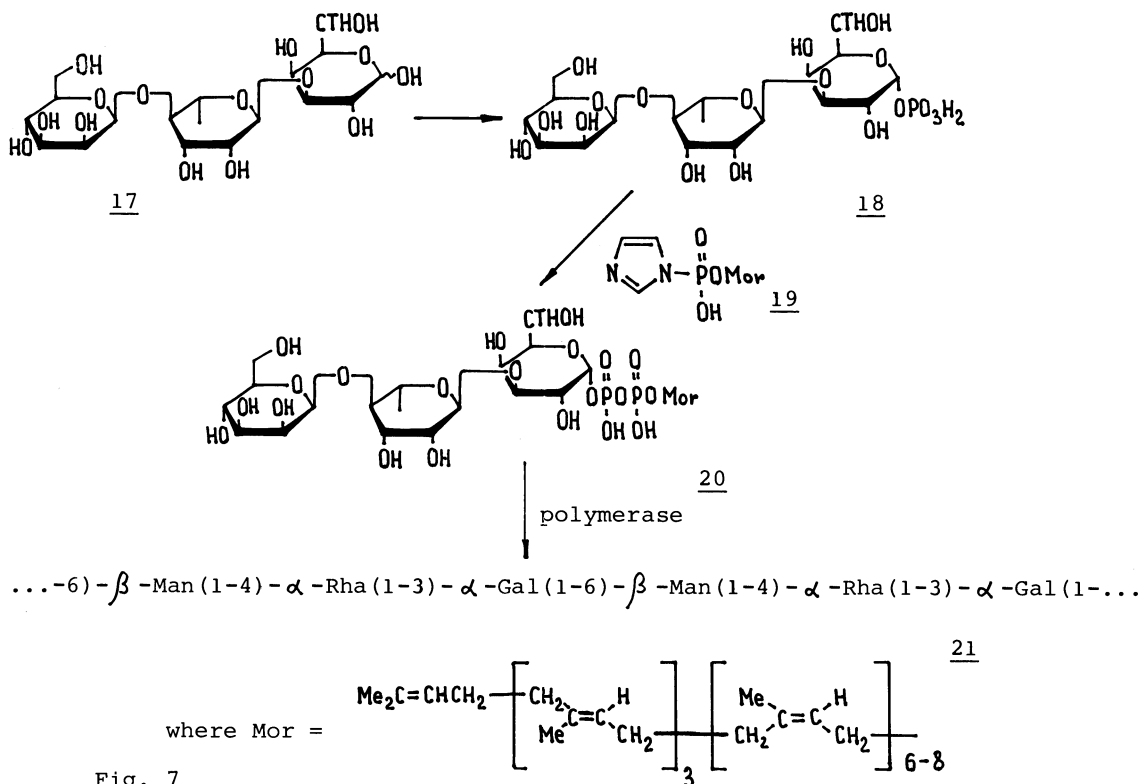which is inaccessible by the chemical polymerization method (Fig. 7).



$...-6)-\beta$-Man(1-4)-$\alpha$-Rha(1-3)-$\alpha$-Gal(1-6)-$\beta$-Man(1-4)-$\alpha$-Rha(1-3)-$\alpha$-Gal(1-...



Fig. 7

For convenient assay of the polymerization reaction a tritium label was in-
corporated 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 phos-
phoric 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-phenyle-
ne 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) pro-
cedure 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 phosphoimidazolidates (Ref. 19), which allo-
wed 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 1-0-( $\alpha$ -galactosyl)-glycerol after Smith degradation, as well as enzymatic hydrolysis with $\alpha$-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-xylo-hexose, 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 op-
posed to T-independent natural carbohydrate antigens (Ref. 24). Finally, since
the attachment of the hapten by existing methods proceeds mainly through the
$\varepsilon$-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 mo-
dified by means of introduction of cross-linkages. The polymer with a polyacryl-
amide 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, contai-
ning i) total repeating unit, namely, trisaccharide (1), which is characteris-
tic of Salmonella anatum and S.newington; ii) monofactor determinant of seve-
ral 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 re-
peating unit of S. anatum as a hapten (Ref. 26). Allyl $\beta$-glycoside of the tri-
saccharide (1) was prepared for the copolymerization with acrylamide. Synthe-
sis of the former is presented in Fig. 8.  Allyl $\beta$-galactoside (22) was subjec-
ted to isopropylidenation, acetylation, and mild hydrolysis to give 2,6-diace-
tate of allyl $\beta$-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
$\beta$-galactoside (24). Condensation of (24) with acetobiosyl bromide (25), gene-
rated 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'-tetramethylethy-
lenediamine. 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
$^{13}$C 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-

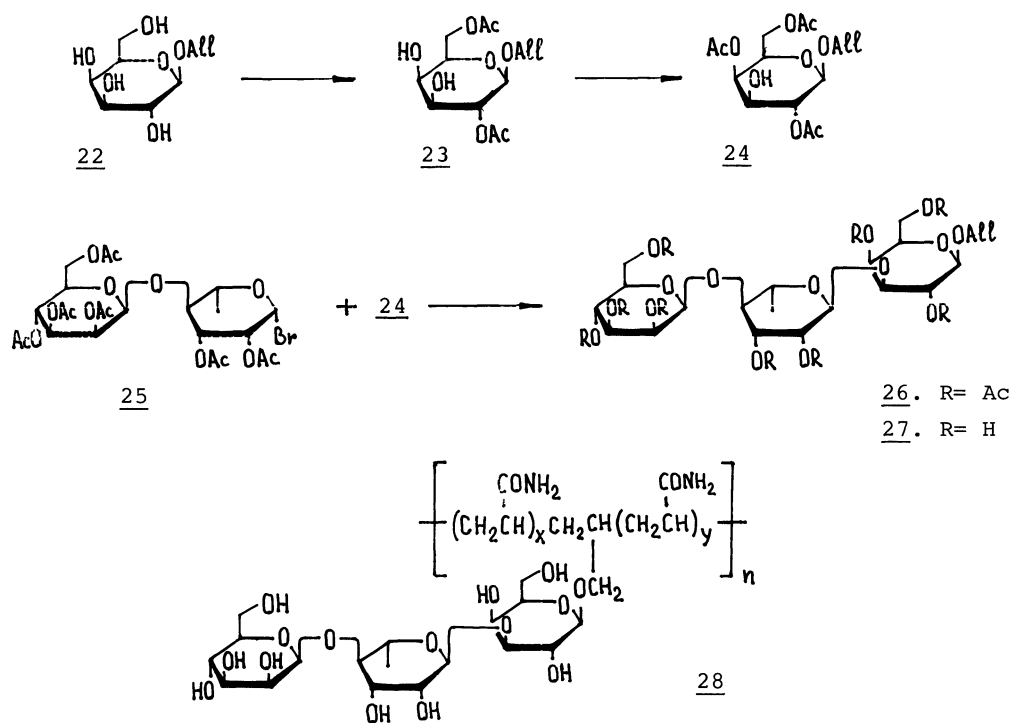tenic groups in (28) within certain limits.



Fig. 8.

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 O: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 haptens 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 O: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 O:4 (abequosyl-mannose hapten), O:9 (tyvelosyl-mannose
hapten), and O:2 (paratosyl-mannose hapten), and the degree of their specifi-
city was tested. Synthesis of the corresponding haptens was performed follo-
wing 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-D-manno-
pyranoside, is shown in Fig. 9. Allyl 2,3:4,6-di-O-isopropylidene-ᵈ-D-manno-
pyranoside (29), prepared by acetonation of allyl ᵈ-D-mannopyranoside, was
subjected to mild acid hydrolysis in aqueous acetone. The resulting 2,3-O-iso-
propylidene derivative (30) was acetylated, de-isopropylidenated, and the
4,6-diacetate (31) through 2,3-orthoacetate, was converted into 2,4,6-triace-
tate of allyl ᵈ-D-**mannopyranoside** (32), **which was** subjected to Helferich gly-
cosylation by **2**,4-di-C-(p-nitrobenzoyl)-ᵈ-D-abequosyl bromide to give the
disaccharide derivative (33), which, when treated with barium oxide in boiling
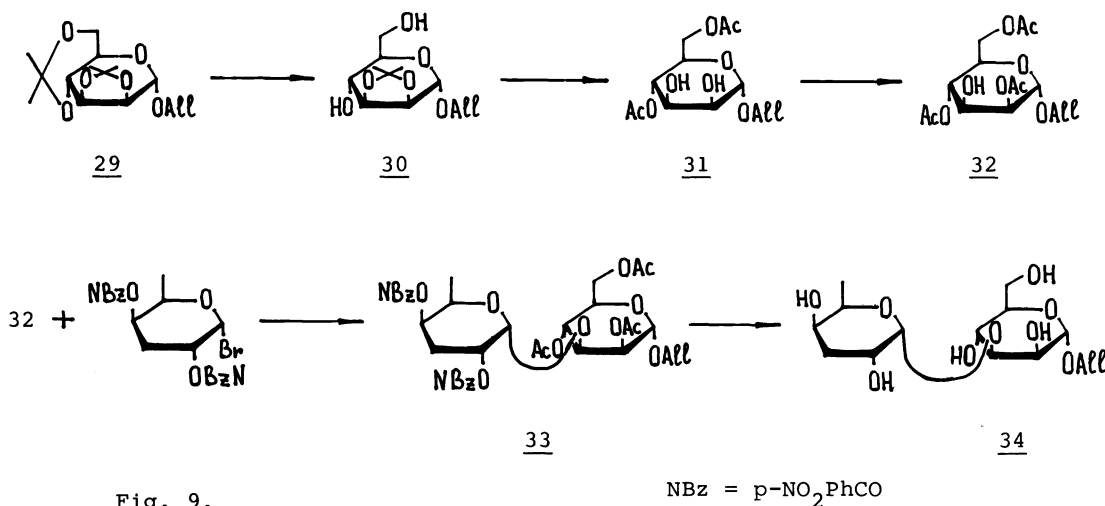methanol,    afforded allyl ᵈ-glycoside of abequosyl-mannose (34).



Fig. 9.                               NBz = p-NO$_2$PhCO

The hapten (34) was copolymerized with acrylamide (in ratio of 2:1) under con-
ditions described above; the copolymer (35) (Fig. 10) of molecular weight
100,000-300,000 was obtained in 25% yield; it contained _ca_. 30% of carbohydra-
tes, 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-manno-
se (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.
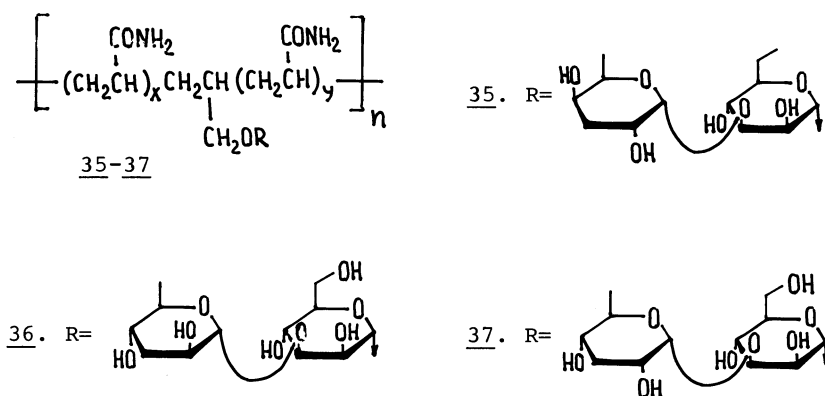


Fig. 10.

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.

$$-3)-\beta\text{-GlcA}(1-4)-\beta\text{-Glc}(1-3)-\beta\text{-GlcA}(1-4)-\beta\text{-Glc}(1- \qquad (38)$$
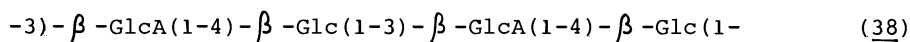
At the same time, the antigenic determinant of this polysaccharide is not strictly known, thus, to prepare the optimum synthetic antigen we had also to solve another problem, namely, to determine the antigenic site of Str. pneumoniae type 3. To this effect, we have synthesized the copolymer involving 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.
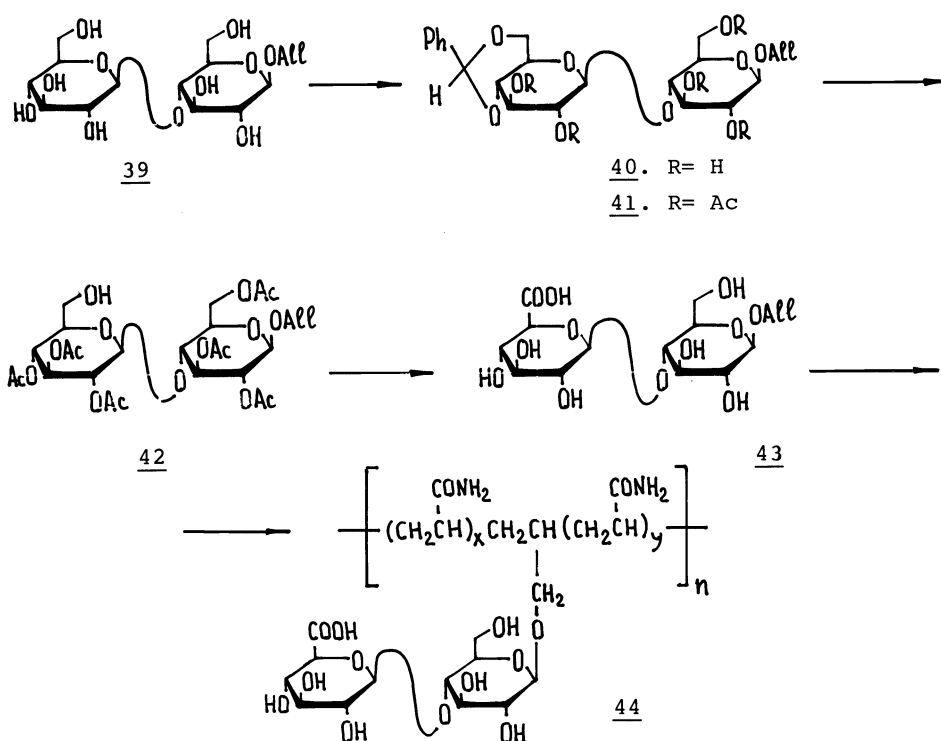
Fig. 11.

The synthesis of the haptenic component for the synthetic antigen containing
pseudoaldobiouronic acid residues, is shown in the Fig. 12. Methyl 5-O-acetyl-
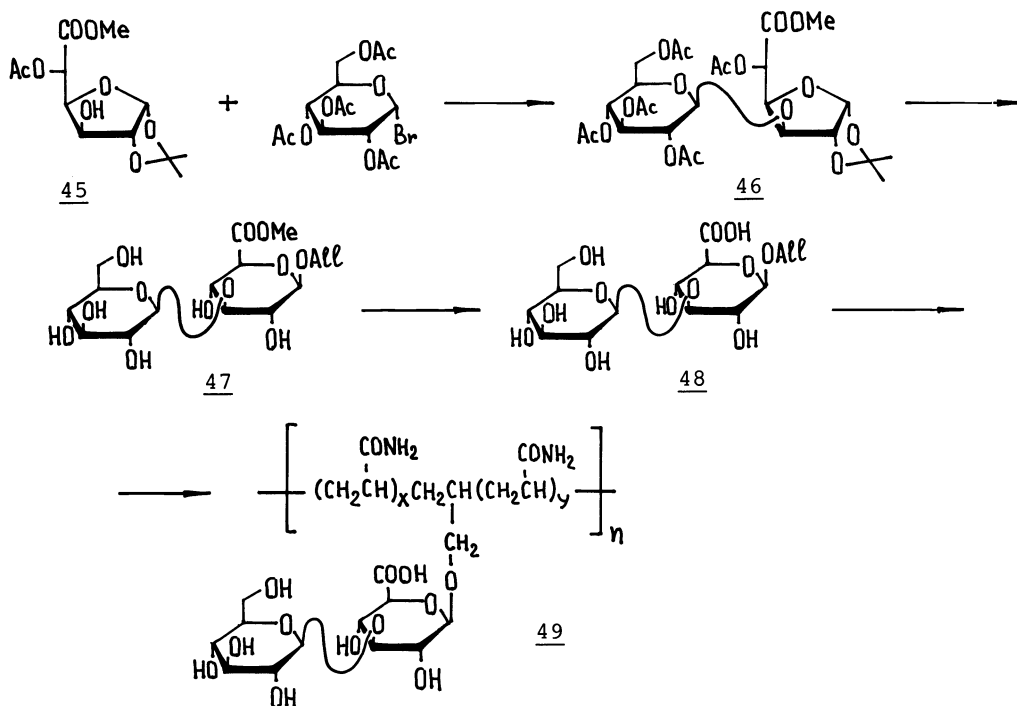-1,2-O-isopropylidene-α-D-glucofuranuronate (45), prepared by known methods

Fig. 12.

from D-glucuronolactone, was glycosylated by acetobromoglucose under Helferich

conditions, $\beta$ -anomer of the pseudoaldobiouronic acid derivative (46) was de-
protected and converted via acetylbiosyl halide into allyl glycoside (47),
the methyl ester was hydrolyzed by 2% KOH in aqueous alcohol at room tempera-
ture to give the haptenic component (48) for the synthetic antigen. Copolyme-
rization of (48) with acrylamide afforded the copolymer (49) which is closely
related to the previous one: molecular weight is 100,000 - 300,000, carbohydra-
te content ca. 30%, hapten : acrylamide ratio is 1:10. Presently, the synthe-
sis is close to completion of the haptenic component bearing the residue (50).

$$\beta\text{-GlcA}(1-4)-\beta\text{-Glc}(1-3)-\beta\text{-GlcA} \qquad\qquad\qquad (50)$$
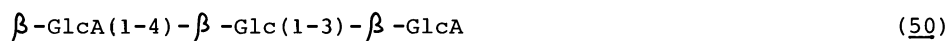
The synthesis consists in glycosylation of the aglycon synthon (45) by glyco-
syl bromide derived from cellobiouronic acid, followed by conversion of the
resulting trisaccharide into allyl $\beta$ -glycoside and copolymerization of the
latter with acrylamide.

Serological specificity of the prepared synthetic antigens was tested by the
ELISA technique for the copolymer with cellobiouronic acid residue as a hap-
ten. The copolymers containing 27 and 13% of carbohydrates exhibited distinct
reaction with antisera against Str. pneumoniae of the serotype 3. They exhibi-
ted 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, out-
lined in this report, appears to be quite general, this is also suitable for
the preparation of capsular carbohydrate antigens.

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