Pure & Appl. Chem., Vol. 50, pp.1421-1436. 0033-4545/78/1201-1421 $02.00/0
Pergamon Press Ltd. 1978. Printed in Great Britain.

BIOSYNTHESIS OF SALMONELLA O-ANTIGENIC POLYSACCHARIDES: SPECIFICITY OF GLYCOSYL TRANSFERASES

Vladimir N. Shibaev

N.D.Zelinsky Institute of Organic Chemistry, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

Abstract - An approach to the study of the specificity of glycosyl transferases is discussed which is based on the use of synthetic substrate analogs. A new method has been developed for the synthesis of polyprenyl pyrophosphate sugars, intermediates in the biosynthesis of Salmonella O-specific polysaccharides. Some synthetic analogs of guanosine diphosphate D-mannose and thymidine diphosphate L-rhamnose have been prepared and their efficiency as substrates for the glycosyl transferases involved in S.anatum O-specific polysaccharide biosynthesis studied. Rather wide specificity of the glycosyl transferases towards the structure of the glycosyl donors is noted and the use of sugar nucleotide analogs for the preparation of modified oligo- and polysaccharides is suggested.

INTRODUCTION

It is generally agreed that the structure of carbohydrate chains in biopolymers is dictated by the specificity of the glycosyl transferases which participate in the biosynthetic process. Each of these enzymes is able to recognise specifically structures of the glycosyl donor and the glycosyl acceptor and catalyse the formation of a specific type of glycosidic linkage, i.e. it can glycosylate a particular hydroxyl group in a polyfunctional acceptor molecule with the formation of an α- or β-glycosidic linkage.

The mechanism of the enzyme-substrate recognition for the glycosyl transferases remains unclear. Generally speaking, enzyme-substrate recognition may depend on several types of interactions. Such recognition may be connected with an interaction between ionic or polar groups of an enzyme and a substrate, particularly by specific hydrogen bonding. Alternatively, it may depend on hydrophobic interactions of a non-polar grouping of a substrate with certain sites on the enzyme surface. Furthermore, formation of an enzyme-substrate complex may be sterically hindered for some substrate analogs with bulky substituents in certain parts of the molecule and such hindrance may also serve as a basis for specific enzyme-substrate recognition. It seems impossible to predict a priori the significance of every type of interaction for such complicated molecules as carbohydrate derivatives.

The problem may be attacked by designing a special set of analogs of the substrate each of which is modified in only one specific site and by a study of the properties of these analogs as substrates or inhibitors of the enzymic reaction.

We have used such an approach in our previous work on the evaluation of the structural features of the uridine diphosphate D-glucose (UDPGlc) molecule for interaction with different enzymes (for a review see Ref. 1). The results are summarised in Fig. 1. The recognition sites for different enzymes are similar in some respects as they always include two groups capable of hydrogen bonding, namely the NH group of the uracil nucleus and HO-3 of the hexosyl residue. For some enzymes the recognition site contains also HO-4 or HO-6 of the hexosyl residue. Modification of the sugar nucleotide at the recognition site groups results in UDPGlc analogs which are unable to act as substrates for enzymic reactions whereas modifications at some other points (shaded areas in Fig. 1) lead to analogs which are rather efficient substrates for the enzymes. It seems probable that hydrogen bonding is of the utmost importance for UDPGlc recognition with the enzymes studied although other mechanisms may also be of some significance.

In recent years we have become interested in the use of the one point-modified-analog approach for investigation of the specificity of glycosyl transferases which participate in the biosynthesis of carbohydrate chains in biopolymers. No systematic studies have been reported in this field. We believe that the enzymes involved in the biosynthesis of Salmonella O-specific polysaccharides represent one of the most convenient systems for this purpose.

SALMONELLA O-SPECIFIC POLYSACCHARIDES: STRUCTURE AND BIOSYNTHESIS

Lipopolysaccharides are normal components of the outer membrane of Gram-negative bacteria. It is well known that these macromolecules may be sub-divided into three parts, namely, the hydrophobic lipid A region, the oligosaccharide core sequences which are similar or identical in lipopolysaccharides of different bacteria, and the O-specific polysaccharide chains. These chains are different in structure for micro-organisms of the same genus and the diversity of their structures determines the serological specificity of the micro-organisms. Serological classification of bacteria of Salmonella genus has been worked out very thoroughly and, in many cases, the structures of O-specific polysaccharides and their antigenic determinants have been completely elucidated (for a comprehensive review see Ref. 2). Exact knowledge about the complete structure of the biosynthetic products and the availability of a wide range of similar polysaccharides from different bacterial strains are advantages of the Salmonella O-specific polysaccharides in a study of the chemical aspects of polysaccharide biosynthesis.

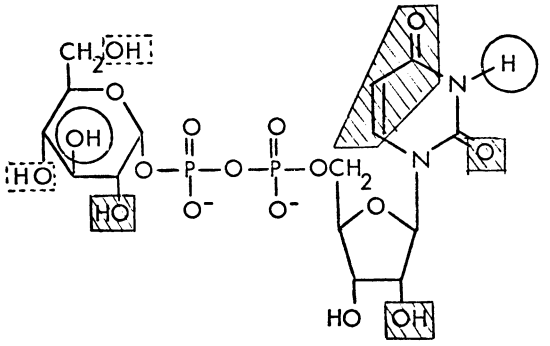


Fig. 1. Evaluation of the different structural factors of the UDPGlc molecule for interaction of the sugar nucleotide with different enzymes. Universal recognition sites are enclosed in circles, dotted lines enclose groups of the recognition sites which are involved only in some cases. The hatched areas indicate the groups which are not part of the recognition sites for the investigated enzymes.

For Salmonella, the structures of many O-specific polysaccharides are closely related. In bacterial strains which belong to the serological groups A, B, D, and E, the main chain of the polysaccharide is composed of repeating D-mannosyl-L-rhamnosyl-D-galactosyl sequences with different types of Gal-Man and Man-Rha linkages in different species (Fig. 2). In some polysaccharides D-mannosyl residues carry 3,6-dideoxyhexose residues of different configuration as branches and D-glucosyl branches may be attached to D-galactosyl residues. The oligosaccharide repeating units are frequently modified by the introduction of acetyl substituents. The major part of our investigations was performed with the S. anatum strain which belongs to serological group E1; the structure of its O-specific polysaccharide (Refs. 3, 4) is based on the trisaccharide repeating unit (Fig. 2b).

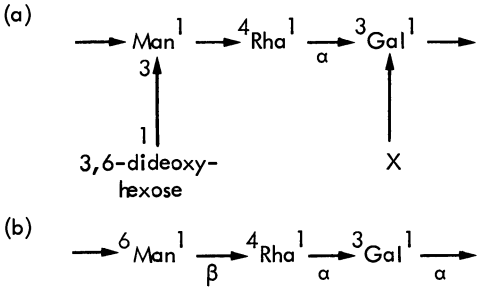


Fig. 2. Structures of the O-specific polysaccharides: (a) general structure for the polymers from Salmonella species of serological groups A, B, D, and E; (b) the polymer from S. anatum.

Another important feature of Salmonella O-specific polysaccharides is that the biosynthetic pathway has been well investigated in several cases. Fig. 3 shows the reaction sequence which takes place in the biosynthesis of O-specific polysaccharide of S. anatum (Ref. 5). The trisaccharide repeating unit is

assembled by consecutive transfers of an α-D-galactopyranosyl phosphate residue from uridine diphosphate D-galactose (UDPGal), an α-L-rhamnopyranosyl residue from thymidine diphosphate L-rhamnose (dTDPRha), and a β-D-mannopyranosyl residue from guanosine diphosphate D-mannose (GDPMan) onto bacterial undecaprenyl phosphate (BPR). The resulting polyprenyl pyrophosphate trisaccharide serves as a substrate for O-antigen polymerase and the polymerisation is followed by transfer of the O-specific chain to the core sequence of the lipopolysaccharide.

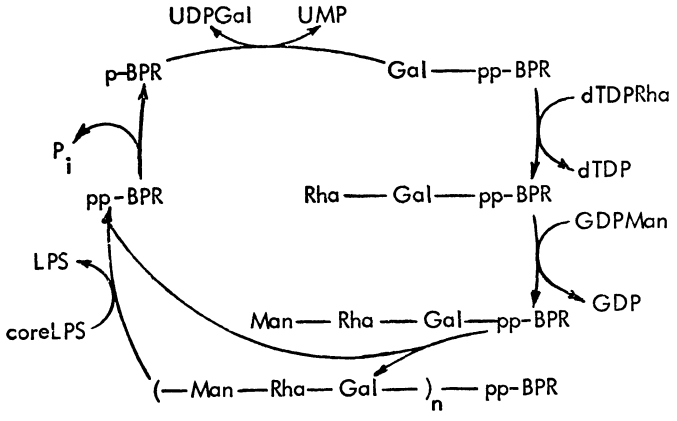


Fig. 3. Pathway of biosynthesis of the O-specific polysaccharide in S. anatum.

A similar sequence of enzymatic reactions has been demonstrated for some other O-specific polysaccharides (Table 1) including polymers from Salmonella species which belong to serological groups E_2 and B. Recently we were able to show that an analogous mechanism of biosynthesis is characteristic for strains of serological groups E_4, C_2, and C_3. In the last two cases, transfer of a D-galactopyranosyl phosphate is followed by transfer of two D-mannose, D-glucose and L-rhamnose residues; the incorporation of 3,6-dideoxyhexosyl residues was not investigated.

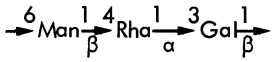
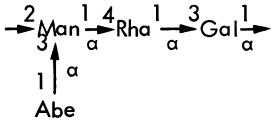
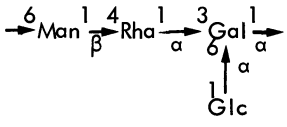
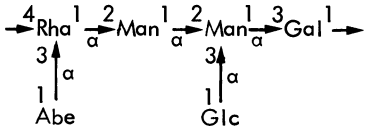
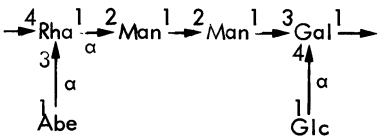
The first reaction of the biosynthetic sequence is the formation of polyprenyl pyrophosphate galactose for all the polymers which were investigated. It seems that D-galactose is the preferred monosaccharide residue on the reducing end of the biological repeating unit of the polysaccharide. It may be even that the presence of D-galactose in a polysaccharide is a necessary prerequisite for the functioning of the above mechanism of biosynthesis of a carbohydrate chain. Recent results of Kopmann and Jann on Escherichia coli 08 polysaccharide (Ref. 14) and Nikaido (personal communication) on the O-specific polysaccharide of S. montevideo (serological group C_1) demonstrates that biosynthetic mechanisms of other types operate for polymers which do not contain D-galactose residues.

Therefore, O-specific polysaccharides of different structures but with similar pathways of biosynthesis arise because of the different substrate specificities of the glycosyl transferases which catalyse the transfer of the second, the third etc. monosaccharide residues onto the polyprenyl pyrophosphate galactose and the specificity of the O-antigen polymerases which catalyse the conversion of polyprenyl pyrophosphate oligosaccharides into the polysaccharide.

CHEMICAL SYNTHESIS OF POLYPRENYL SUGAR INTERMEDIATES IN THE BIOSYNTHESIS OF THE O-SPECIFIC POLYSACCHARIDES

Glycosyl transferases which participate in the biosynthesis of Salmonella O-specific polysaccharides use polyprenyl pyrophosphate galactose or polyprenyl pyrophosphate oligosaccharides with D-galactose on the reducing end of the carbohydrate chain as glycosyl acceptors. We have been interested in the chemical synthesis of such derivatives and their analogs for studying acceptor specificity of the glycosyl transferases and O-antigen polymerase.

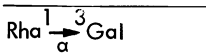
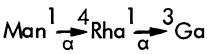
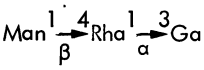
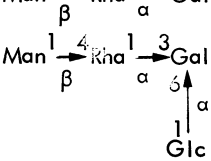
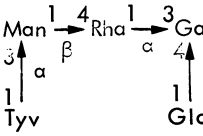
TABLE 1. Structures of Salmonella O-specific polysaccharides with known biosynthetic pathways

Polysaccharide structure (OAc groups are omitted)	Bacterial species	Serological group	Refs. Structure	Refs. Biosynthesis
	S. newington	E_2	3,4,6	5
	S. typhimurium	B	4,7-9	10
	S. seftenberg	E_4	11	*
	S. newport	C_2	12	*
	S. kentucky	C_3	13	*

* V. N. Shibaev, T. N. Druzhinina, A. N. Popova, S. S. Rozhnova, and V. A. Kilesso, unpublished results.

An extensive program of chemical synthesis of the oligosaccharide fragments of Salmonella O-specific polysaccharides with D-galactose at the reducing end and of their analogs is now under way in the laboratory of Professor N. K. Kochetkov in Moscow. The structures of some of the fragments which have been synthesised are shown in Table 2 including di-, tri-, tetra-, and pentasaccharide derivatives.

TABLE 2. Structures of some synthetic oligosaccharide fragments of Salmonella O-specific polysaccharides

Oligosaccharide structure	Serogroup	Refs.
	A,B,D,E	15
	A,B,D_1	16
	D_2,E_1-E_4	17-19
	E_4	20
	D_2	*

* N. K. Kochetkov, N. N. Malysheva, and V. I. Torgov, unpublished results.

The synthetic schemes involved may be adapted or modified for the preparation of the radioactively labeled oligosaccharides. Fig. 4 shows the synthesis of the trisaccharide fragment of the Salmonella O-specific polysaccharides from serological groups D_2 and E which contains a radioactive label at C-2 of the D-mannosyl residue (Ref. 21) incorporated by reduction of the disaccharide ulose derivative with sodium borotritiide.

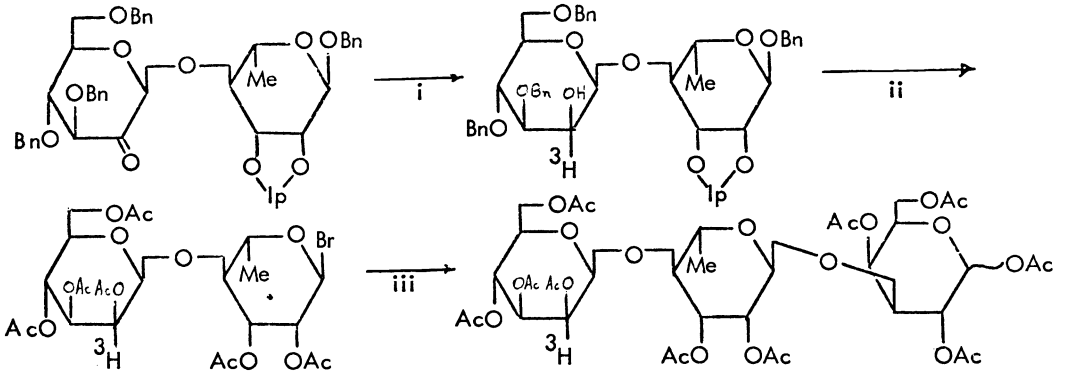


Fig. 4. Synthesis of the trisaccharide derivative with radioactive label in D-mannose residue. (i) NaB^3H_4, (ii) H_2/Pd, Ac_2O/Py, CF_3CO_2H, Ac_2O/Py, HBr/AcOH; (iii) Koenigs-Knorr with benzyl 2,6-di-O-acetyl-β-D-galactopyranoside, Et_3N/MeOH, Ac_2O/Py, H_2/Pd, Ac_2O/Py.

The successful synthesis of the oligosaccharide fragments of Salmonella O-specific chains allowed us to begin a study of the synthesis of the polyprenyl pyrophosphate sugar intermediates of the O-specific polysaccharides. Such a study should include (a) syntheses of oligosaccharide phosphates which may be referred to as substituted α-D-galactopyranosyl phosphates, (b) preparation or isolation of bacterial undecaprenyl phosphate or its analog which may serve as a substrate for the enzymes of the O-specific polysaccharide biosynthesis, and (c) development of efficient methods for the chemical synthesis of polyprenyl pyrophosphate D-galactose and related derivatives of oligosaccharides. The results of our investigations along these lines are now presented.

The most straightforward method for the preparation of glycosyl phosphates is the MacDonald procedure, namely, phosphorylation of sugar acetates with anhydrous phosphoric acid; the method is used frequently for the synthesis of monosaccharide phosphates (for a review see Ref. 22). We have shown that the procedure may be successfully applied to disaccharides such as maltose and cellobiose (Ref. 23). Our more recent data (V. N. Shibaev, M. F. Troitzky, L. L. Danilov, and V. N. Chekunchikov, unpublished results) indicate that the disaccharide and the trisaccharide fragments of the O-specific polysaccharides may be also efficiently phosphorylated by this method (Fig. 5). We have not observed any significant phosphorolysis of the acid-labile α-rhamnosyl and β-mannosyl linkages which are present in the oligosaccharides and the oligosaccharide phosphates were isolated after deacetylation in fair yields. The high positive optical rotation of the oligosaccharide phosphates and the decrease on removal of the phosphate group with phosphomonoesterase indicates the α-configuration of the glycosyl-phosphate linkage, this conclusion is supported by p.m.r. data for the disaccharide derivative.

The second component which is required for synthetic studies of polyprenyl pyrophosphate sugars is a polyprenyl phosphate. The isolation of bacterial undecaprenyl phosphate has been described (Ref. 24) but its preparation in amounts sufficient for synthetic studies through a rather tedious procedure remains questionable. Readily available plant polyprenols are much better starting materials. These polyprenols are similar to but not identical in structure with the bacterial polyprenols (for a review see Ref. 25). Generally, the ratio of E- and Z-isoprenic units was found to be 2:8 for bacterial undecaprenols whereas, for ficaprenol-11 and related plant polyprenols, the ratio 3:7 was characteristic. Nevertheless, ficaprenyl phosphate was found to substitute for bacterial undecaprenyl phosphate in some membrane-bound enzyme systems and it seems that these enzymes are not strictly specific in respect of chain length and stereochemistry of polyprenols (Ref. 26).

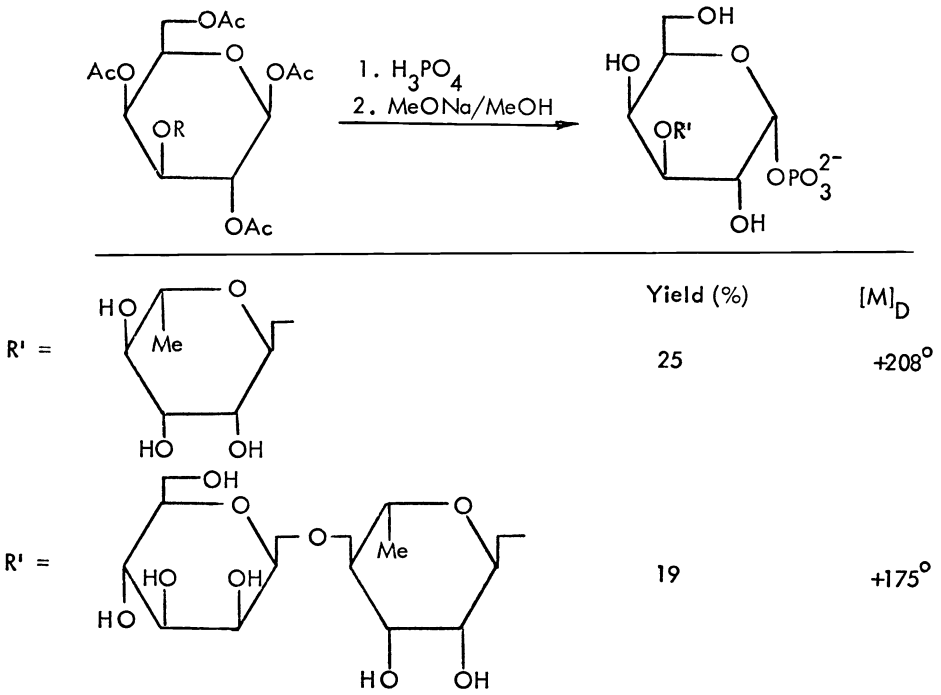


Fig. 5. Synthesis of glycosyl phosphates derived from oligosaccharide fragments of Salmonella O-specific polysaccharides.

Mulberry leaves are one of the most readily available sources of polyprenols. The polyprenols from this plant (moraprenols) were purified and partially characterised by Japanese investigators (Ref. 27). Their structure elucidation was completed in our laboratory (Ref. 28) and ^{13}C-n.m.r. spectroscopy was used to determine the distribution of E- and Z-isoprenic units along the polyprenol chain. The structure of moraprenol-11 is shown in Fig. 6 in comparison with the structure suggested for bacterial undecaprenol. The most widely used plant polyprenols, ficaprenols, are probably identical with moraprenols, but to my knowledge the distribution of isoprene units of different stereochemistry along the ficaprenol chain has never been determined unequivocally.

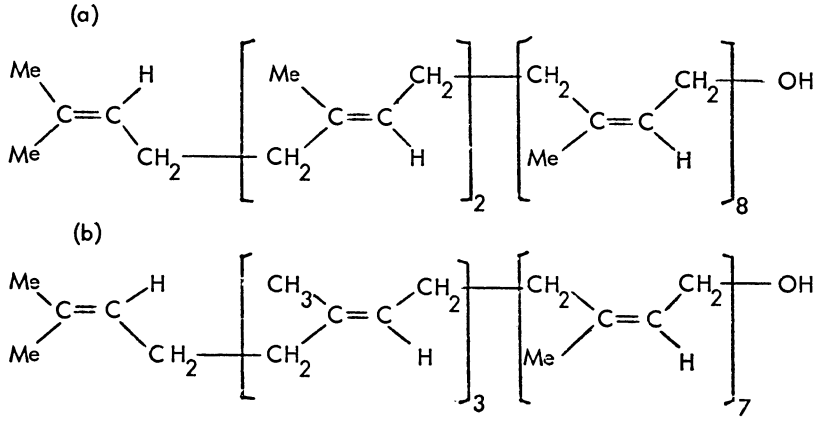


Fig. 6. Structure of polyprenols: (a) bacterial undecaprenol (suggested); (b) moraprenol-11.

Moraprenol was converted into moraprenyl phosphate by phosphorylation with o-phenylene phosphochloridate followed by removal of the protecting group with lead tetra-acetate and alkali (Fig. 7). The procedure for polyprenol phosphorylation originally developed by Warren and Jeanloz (Ref. 29) was modified to increase the yield and purity of the product (Ref. 28). Moraprenyl phosphate was found to be a rather efficient substitute for bacterial undecaprenyl phosphate in the first reaction of the O-antigen biosynthesis in S. anatum (see Fig. 3) and the moraprenyl pyrophosphate galactose formed may enter the following reactions of the biosynthetic cycle (Ref. 30). These results show that moraprenyl pyrophosphate sugars are biologically active analogs of the corresponding bacterial undecaprenol derivatives and their chemical synthesis is worthwhile.

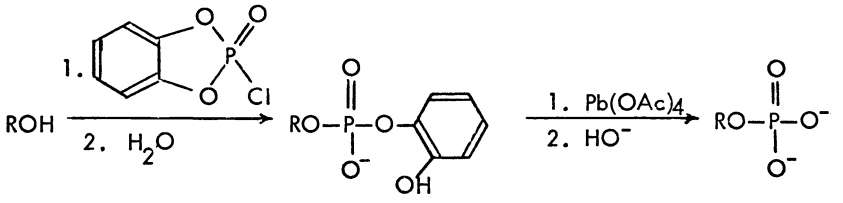


Fig. 7. Synthesis of moraprenyl phosphate.

The first syntheses of polyprenyl pyrophosphate sugars were performed in the laboratory of Dr R. W. Jeanloz in Boston, namely, derivatives containing α-D-galactopyranosyl (Ref. 29), α-D-mannopyranosyl (Ref. 31) and 2-acetamido-2-deoxy-α-D-glucopyranosyl (Refs. 32, 33) residues. The method used involved the interaction of P^1-polyprenol, P^2-diphenyl pyrophosphates prepared from polyprenyl phosphates and diphenyl phosphochloridate with fully acetylated glycosyl phosphates as illustrated in Fig. 8. The protected pyrophosphate derivatives were deacetylated with MeONa in MeOH which gave clear cut results in the last two cases. In contrast, heavy losses of product may occur for the α-D-galactopyranosyl pyrophosphate derivative, which is highly unstable to base treatment due to participation of HO-2 in the cleavage of the pyrophosphate linkage.

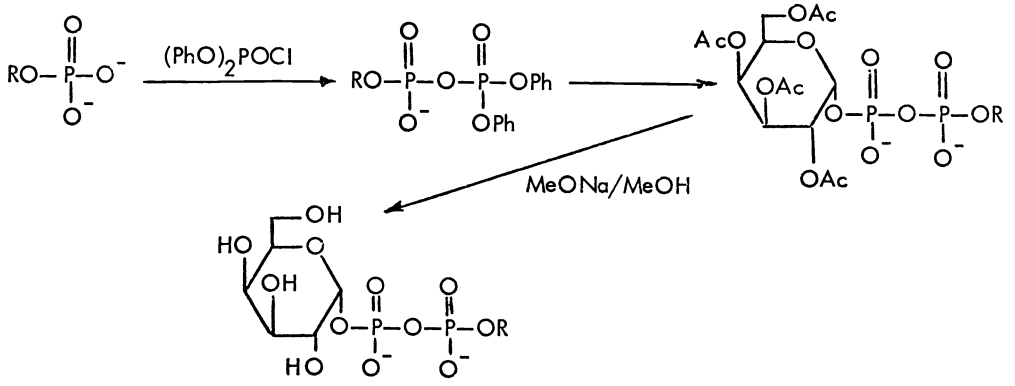


Fig. 8. Synthesis of ficaprenyl pyrophosphate D-galactose according to Warren and Jeanloz (Ref. 29).

The need for protection of the sugar hydroxyl groups in the pyrophosphate synthesis is not obvious as similar reactions in nucleoside pyrophosphate sugar syntheses proceed smoothly with unprotected glycosyl phosphates. As mentioned before, the intermediates of Salmonella O-specific polysaccharide biosynthesis are always polyprenyl pyrophosphate D-galactose derivatives and development of a synthetic method for these compounds which did not include base treatment as the last step was highly desirable. The problem was solved by using polyprenyl phosphoimidazolidates as activated components in the pyrophosphate synthesis (Fig. 9) (V. N. Shibaev, L.L. Danilov, V. N. Chekunchikov, unpublished results). Moraprenyl phosphate was converted into the phosphoimidazolidate by treatment with N,N'-sulfonyldiimidazole prepared in situ from sulfonyl chloride and imidazole (Ref. 34). This reagent was used previously for the synthesis of some analogs of nucleoside triphosphates (Ref. 35). After destroying the excess of the activating agent the phosphoimidazolidate was reacted without purification with trioctylammonium α-D-galactopyranosyl phosphate giving moraprenyl pyrophosphate D-galactose which was isolated in high yield after purification by ion-exchange chromatography. The structure of the product was confirmed by the

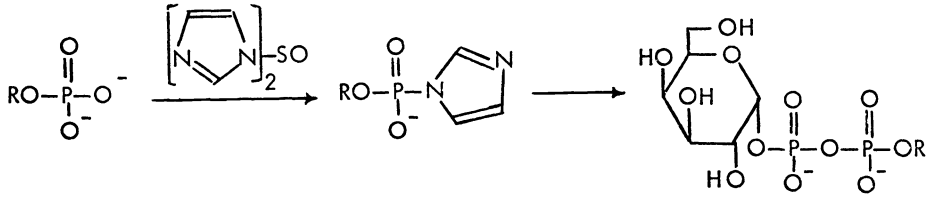


Fig. 9. Synthesis of moraprenyl pyrophosphate D-galactose.

characteristic degradation products shown in Fig. 10. Moreprenyl phosphate and the sugar 1,2-cyclic phosphate were identified after short treatment with ammonia whereas reaction with aqueous phenol gave the glycosyl pyrophosphate. The latter product may be dephosphorylated with phosphomonoesterase to identify the sugar residue.

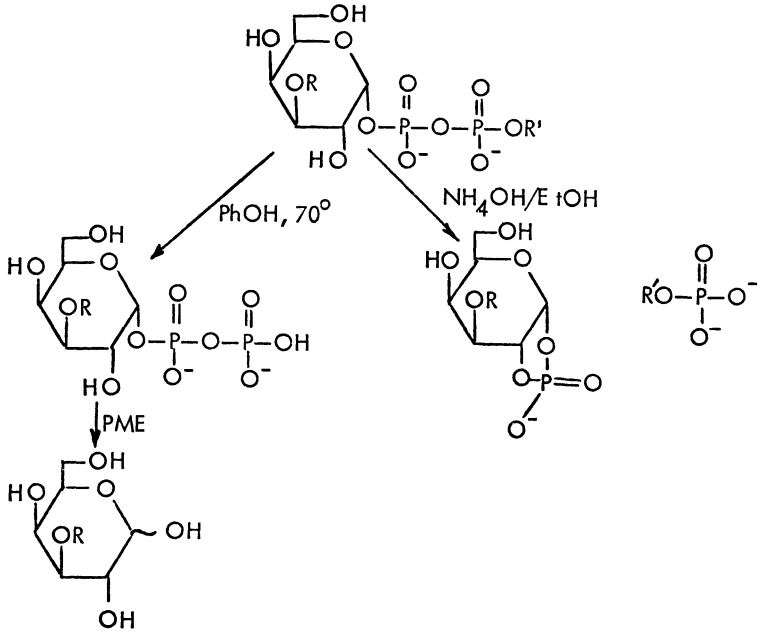


Fig. 10. Degradation products of moraprenyl pyrophosphate sugars.

The first experiments on the application of the method to oligosaccharide phosphates have given promising results. We hope that preparation of the polyprenol-linked oligosaccharide intermediates of Salmonella O-specific polysaccharides and their analogs will be possible in the near future and thereby allow a study of the specificity of the enzymes which participate in O-antigen biosynthesis.

SOLUBILIZATION AND PURIFICATION OF THE ENZYMES OF THE O-SPECIFIC POLYSACCHARIDE BIOSYNTHESIS

The biosynthesis of bacterial lipopolysaccharides including the formation of the O-specific chains occurs inside the inner cytoplasmic membrane and the enzymes of the cycle shown in Fig. 3 are tightly bound to the cell envelope fraction. The galactosyl phosphate transferase and some glycosyl transferases which participate in the assembly of the repeating unit of the O-specific polysaccharide may be solubilised by treatment of cell envelope preparations with non-ionic detergents (Refs. 24, 30). The soluble preparation does not contain the O-antigen polymerase, but that from S. anatum catalyses the formation of the polyprenyl pyrophosphate trisaccharide from the polyprenyl phosphate and the respective sugar nucleotides. The enzymes are rather stable in this preparation which may be easily used to study the donor specificity of glycosyl transferases by the addition of sugar nucleotide analogs to the system instead of the natural glycosyl donors.

Progress in the separation and purification of individual enzymes associated with O-specific polysaccharide biosynthesis is still very limited. We were most successful in the purification of the galactosyl phosphate transferase from S. anatum, the first enzyme of the biosynthetic cycle. As for glycosyl transferases from mammalian tissues, affinity chromatography with immobilised nucleotides was found to be the most useful technique for this purpose. A new type of affinity adsorbent containing nucleotide residues linked to the matrix through a P-N-linkage was used in our studies. These adsorbents are readily available (Ref. 36) as shown in Fig. 11 for the uridine 5'-phosphate derivative. A similar reaction sequence was employed for the preparation of the adsorbents with uridine 5'-pyrophosphate ligands.

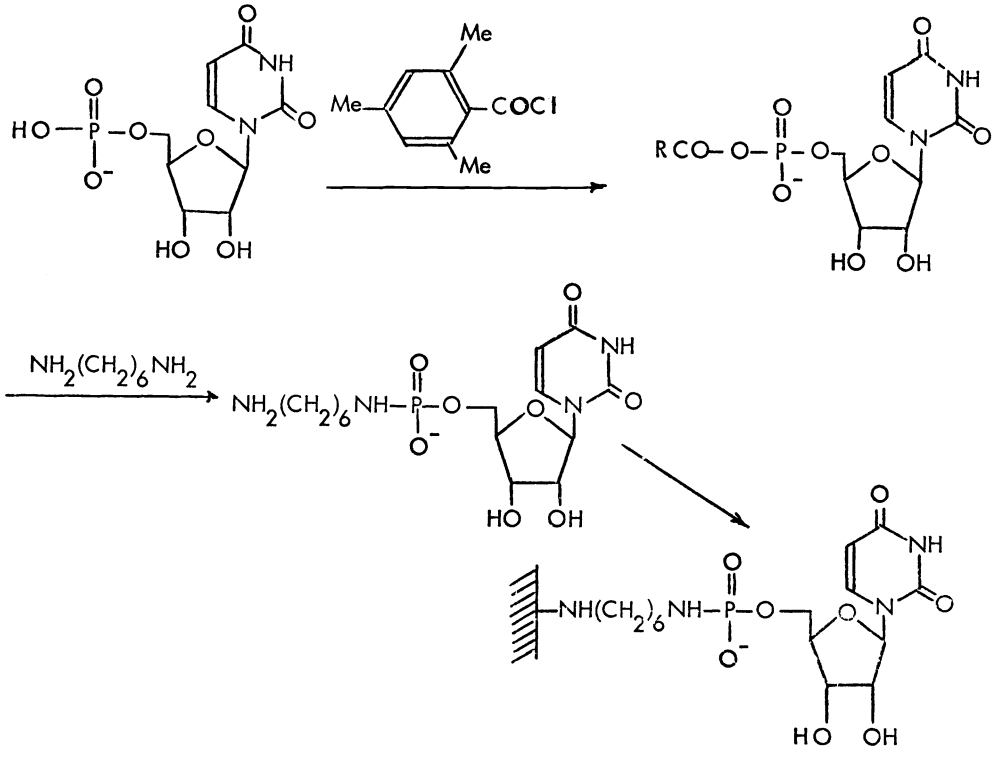


Fig. 11. Preparation of affinity adsorbents with uridine 5'-phosphate ligands.

Application to the affinity column of the soluble enzyme preparation, obtained by the use of a low concentration of detergent resulted in complete adsorption of the galactosyl phosphate transferase. Desorption with UDPGal solution gave an enzyme preparation which was homogeneous on polyacrylamide gel electrophoresis (Ref. 37). Further experiments are in progress in this direction.

SYNTHETIC ANALOGS OF GUANOSINE DIPHOSPHATE D-MANNOSE AS SUBSTRATES OF MANNOSYL TRANSFERASE FROM S. anatum

Evaluation of the structural features of sugar nucleotides in relation to interaction with glycosyl transferases may result from investigation of substrate or inhibitor properties of sugar nucleotide analogs. An extensive set of substrate analogs is required in order to obtain a full picture but even the first results of such a study in a previously completely unexplored field are of interest and our present knowledge on glycosyl donor specificities of mannosyl and rhamnosyl transferases from S. anatum will now be discussed.

Six analogs of guanosine diphosphate D-mannose have been studied as substrates of the mannosyl transferase. Three of them contained modified nucleoside residues (Fig. 12), namely, derivatives of inosine, 1-N-methylguanosine and 8-bromoguanosine (Ref. 38).

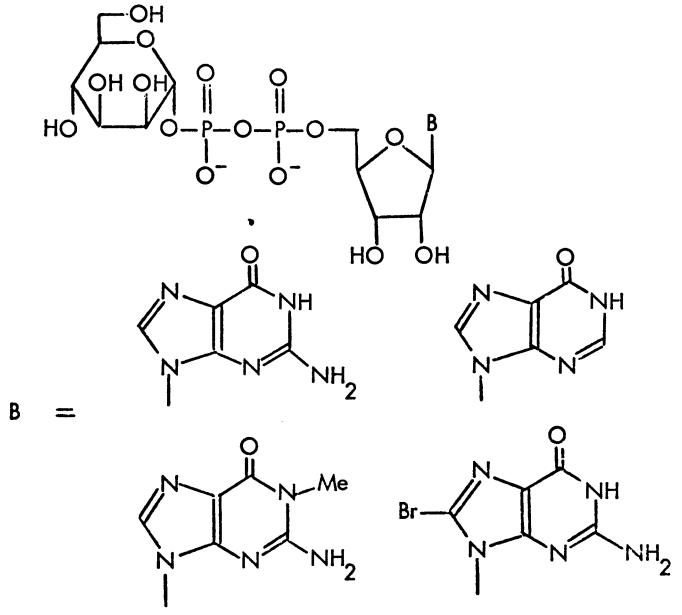


Fig. 12. Analogs of GDPMan with modified nucleoside residue.

The hexosyl residue was modified in three other analogs of GDPMan. Their structures are shown in Fig. 13; HO-6, HO-3, or HO-2 of the D-mannosyl residue was substituted by hydrogen (Ref. 39, also V. N. Shibaev, T. N. Druzhinina, and Yu. Yu. Kusov, unpublished results). The first two analogs of this group were synthesised in the laboratory of Professor Š. Bauer in Bratislava and were kindly supplied to us by Dr Š. Kučar. The 2-deoxy derivative as well as all other sugar nucleotide analogs were prepared by our group using the classical phosphomorpholidate and diphenyl pyrophosphate methods of pyrophosphate synthesis (for a review see Ref. 40).

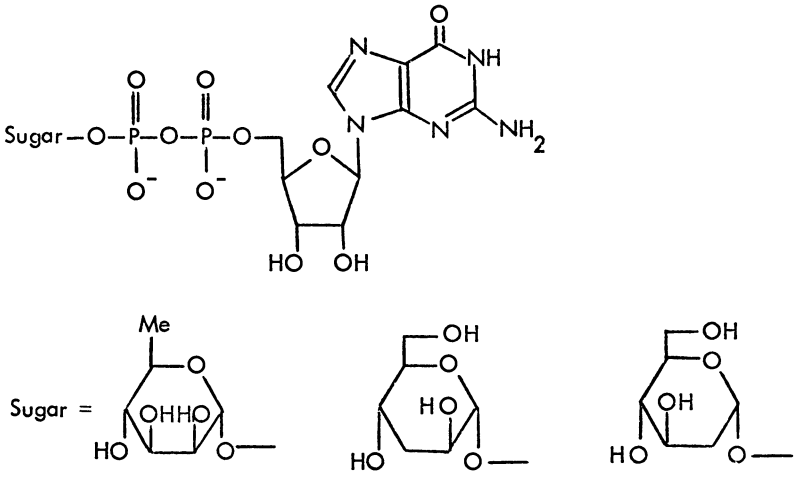


Fig. 13. Analogs of GDPMan with modified hexosyl residue.

In our studies we used a standard system for determining the efficiency of sugar nucleotide analogs as substrates for glycosyl transferases (Ref. 38). In this system relative substrate concentration (σ), relative rate of the reaction (ν_{σ}) and relative Michaelis constant (K_m^r) are defined as follows:

σ = (Concentration of an analog)/(Apparent K_m for the normal substrate)
ν = (Reaction velocity for an analog at the relative concentration shown)/(maximal velocity of the reaction with the normal substrate under assay conditions)
K_m^r = (K_m for an analog)/(K_m for the normal substrate).

The main criterion for evaluation of the substrate efficiency is the value of ν_{10} on the basis of which analogs may be divided into five groups as shown in Table 3 ranging from a very efficient substrate (group 5) to a non-substrate (group 1). A similar classification may be based on the K_m^r values for the analogs.

TABLE 3. Classification of substrate analogs on basis of their efficiency as substrates of the enzymic reactions

Efficiency rank	ν_{10}	K_m^r
5	$\geqslant 0.67$	$\leqslant 5$
4	0.25 - 0.66	5.1-30
3	0.10 - 0.24	31 - 100
2	0.01 - 0.099	101-1000
1	< 0.01	>1000

The results of the assay of analogs of GDPMan with modified nucleoside residues as substrates for mannosyl transferase are summarised in Table 4. The inosine derivative was found to be a very efficient substrate of the reaction, the 1-N-methylguanosine analog may be classified as a good substrate, and the 8-bromoguanosine derivative is a substrate of intermediate efficiency (Ref. 38).

TABLE 4. Substrate properties of GDPMan analogs with modified nucleoside residues for the mannosyl transferase from S. anatum

Nucleoside residue	ν_{10}	K_m^r	Efficiency rank
Inosine	0.67	1.3	5
1-N-Methylguanosine	0.28	25	4
8-Bromoguanosine	0.18	70	3

These results show that neither the NH_2-group nor the NH-group of the guanine ring is significant for the recognition of GDPMan by the mannosyl transferase. All hypothetical possibilities for hydrogen bonding between the guanine nucleus and the enzyme functional groups are shown in Fig. 14. It seems clear that hydrogen bonding in which the heterocycle functions as a donor does not occur in the enzyme-substrate complex of the mannosyl transferase. Thus, the mechanism of recognition of GDPMan by the enzyme is completely different from that which operates for UDPGlc where the NH-group of the ring is a universal group of the recognition sites for different enzymes.

The lowered efficiency of the 8-bromoguanosine derivative as a substrate for the mannosyl transferase is probably connected with the well-known effect of bulky substituents at C-8 of the purine ring on the position of the conformational equilibrium between syn and anti conformers of the nucleoside residue rather than with some kind of specific interaction involving the CH-group of the nucleus. The results may reflect a preference of the enzyme for the anti conformer of the sugar nucleotide although further studies are necessary to substantiate this point.

The mannosyl transferase from S. anatum was found to catalyse the transfer of residues of deoxy-D-mannose analogs (Ref. 39, also V. N. Shibaev, T. N. Druzhinina, and Yu. Yu. Kusov, unpublished results). The results are summarised in Table 5. The transfer is the most efficient for the 6-deoxy derivative, the 3-deoxy and the 2-deoxy derivatives being substrates of intermediate efficiency. Therefore, some analogs of the O-specific polysaccharide fragments with modified D-mannosyl residues may be prepared through enzymatic glycosylation, and they may be used in studies of the relation between structure and activity in serological reactions.

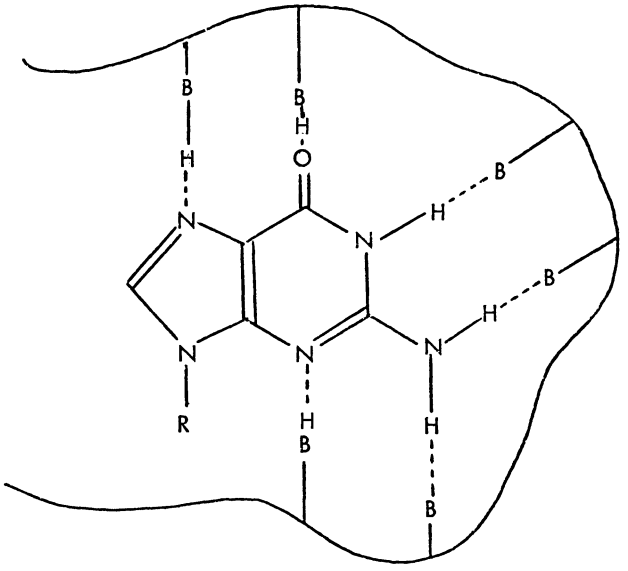


Fig. 14. Possibilities of hydrogen bonding between functional groups of the guanine ring and an enzyme.

The results obtained allow the conclusion that interactions with participation of HO-6, HO-3, or HO-2 of the hexosyl residue are not critical for the specific recognition of GDPMan by the mannosyl transferase. The low significance of HO-3 which is a necessary part of the recognition sites for UDPGlc, is of special interest.

TABLE 5. Substrate properties of GDPMan analogs with modified hexosyl residue for the mannosyl transferase from S. anatum

Hexosyl residue	ν_{10}	Efficiency rank
6-Deoxy-α-D-mannopyranosyl	0.29	4
3-Deoxy-α-D-arabino-hexopyranosyl	0.12	3
2-Deoxy-α-D-arabino-hexopyranosyl	0.11	3

SYNTHETIC ANALOGS OF THYMIDINE DIPHOSPHATE L-RHAMNOSE AS SUBSTRATES OF THE RHAMNOSYL TRANSFERASE FROM S. anatum

In parallel with studies on the specificity of the mannosyl transferase described in the previous section similar studies on the rhamnosyl transferase from S. anatum are under way in our group. Two analogs of thymidine diphosphate L-rhamnose with modified 6-deoxyhexosyl residues were prepared (Refs. 41, 42). The L-mannosyl derivative contains an additional hydroxyl group at position 6 of the monosaccharide residue in comparison with the natural substrate whereas, in the 4,6-dideoxy-β-L-lyxo-hexopyranosyl analog, HO-4 is replaced by hydrogen (Fig. 15).

TABLE 6. Substrate properties for the rhamnosyl transferase from S. anatum of dTDPRha analogs with modified glycosyl residues

Glycosyl residue	Concentration (mM)	σ	ν_σ
β-L-Mannopyranosyl	0.55	32	0.17
4,6-Dideoxy-β-L-lyxo-hexopyranosyl	2.5	150	0.17

The rhamnosyl transferase was found to catalyse the transfer of the modified glycosyl residues (Table 6), although both of the analogs were rather poor substrates and the use of high concentrations were necessary in order to give sufficient product to identify.

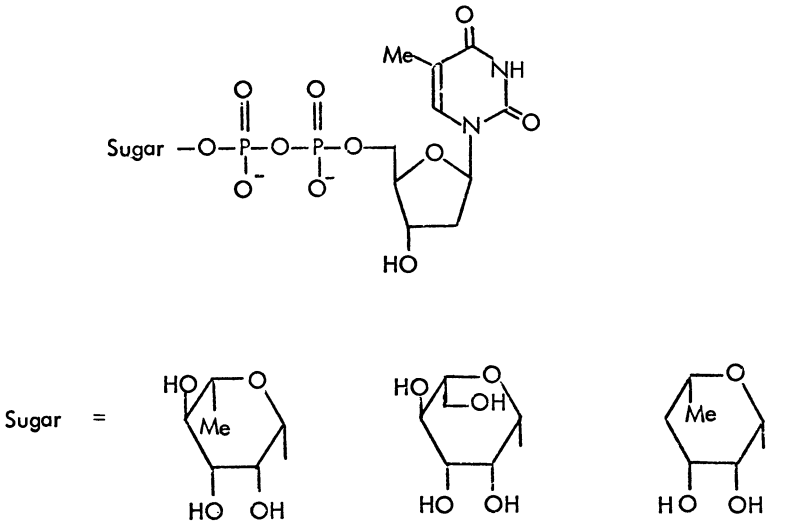


Fig. 15. dTDPRha and its analogs with modified sugar residues.

Furthermore, the polyprenyl pyrophosphate disaccharide formed from thymidine diphosphate L-mannose was found to serve as an acceptor in the subsequent reaction of the cycle with GDPMan. Consequently, the enzymatic synthesis of the analog of the trisaccharide repeating unit of the O-specific polysaccharide which contains D- and L-mannosyl residues was demonstrated.

The ability of analogs of dTDPRha to serve as substrates for the rhamnosyl transferase allows the conclusion that, although interactions of Me-5 (probably hydrophobic) and HO-4 (probably hydrogen bonding) are of significance for interaction of dTDPRha with the enzyme, they do not play a decisive role in substrate recognition. Evaluation of the significance of other functional groups of the L-rhamnosyl residue requires the synthesis and investigation of other analogs.

Another group of dTDPRha analogs was investigated to decide whether the rhamnosyl transferase can distinguish the sugar nucleotides derived from thymidine and uridine. The uridine and 2'-deoxyuridine analogs of dTDPRha (Fig. 16) were prepared for this purpose (Ref. 43).

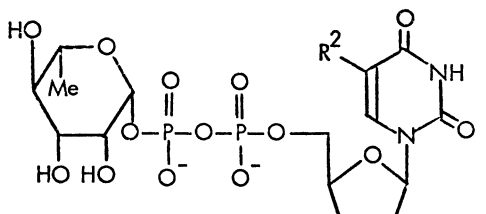


Fig. 16. Analogs of dTDPRha with modified nucleoside residue.

Both analogs were found to be rather efficient substrates of the rhamnosyl transferase (Ref. 44). The data in Table 7 indicate that the presence of a deoxy group at C-2 of the pentosyl residue and an Me group at C-5 of the heterocyclic nucleus are not necessary for interaction of dTDPRha with the rhamnosyl transferase.

TABLE 7. Substrate properties of dTDPRha analogs with modified nucleoside residue for the rhamnosyl transferase from S. anatum.

Nucleoside residue	Concentration (mM)		
2'-Deoxyuridine	0.50	30	0.60
Uridine	0.50	30	0.34

Uridine diphosphate L-rhamnose prepared in our work was previously isolated from several sources, particularly from a Salmonella strain (Ref. 45). The specificity of S. anatum rhamnosyl transferase is broad enough to use this substrate as a glycosyl donor in the biosynthesis of the O-specific polysaccharide. Nevertheless, its functioning as the natural substrate in the biosynthesis seems improbable at least in the strain investigated. Cell-free extracts of the strain catalyse the efficient conversion of thymidine diphosphate D-glucose into thymidine diphosphate L-rhamnose, but are not able to synthesise uridine diphosphate L-rhamnose in an analogous manner (Ref. 44). It follows that substrate specificity of the enzymes which catalyse interconversion of the sugar nucleotides is significantly more strict than that of the glycosyl transferase. Such differences in the breadth of specificity between enzymes of these two types may be a more general phenomenon.

CONCLUSION

The foregoing account illustrates the scope of the one-point-modified analog approach in studies of enzyme-substrate recognition in glycosyl transferases. Many interesting questions in this field are still unanswered and the preparation of many new analogs is still required if the general picture is to be revealed. Nevertheless, some unexpected and important conclusions may be drawn from the preliminary data.

First, the glycosyl transferases investigated have a rather broad specificity towards the structure of the glycosyl donors. It seems that hydrogen bonding involving the hydroxyl groups of the hexosyl residue and functional groups of the heterocyclic ring is of amazingly low significance at least for the interaction of GDPMan with the mannosyl transferase. This raises the question as to how the glycosyl transferases distinguish the different glycosyl donors. It is possible that other types of interactions such as steric hindrance by substituents or hydrophobic interactions are more important for enzyme-substrate recognition in the glycosyl transferases than is hydrogen bonding. It may be significant that these enzymes show a high affinity for hydrophobic components of the membrane and use highly hydrophobic polyprenol derivatives as substrates.

Furthermore, the broad specificity of glycosyl transferases suggests a rather broad use of enzymatic syntheses for preparation of oligo- and polysaccharide analogs which contain modified glycosyl residues. Such derivatives may be of great interest for immunochemical studies. In many cases such syntheses may be superior to chemical syntheses, especially for the incorporation of a modified sugar residue into rather complicated oligosaccharides.

Acknowledgements - I am very grateful to all members of our research group whose names are listed in the bibliography, for their contribution to this work and especially to Professor N. K. Kochetkov for helpful discussions, constant support and encouragement. I am indebted to Dr S. Bauer and Dr S. Kucar who provided GDPMan analogs with modified hexosyl residue and to Dr H. Nikaido for access to unpublished results on the biosynthesis of Salmonella polysaccharides.

REFERENCES

1. N. K. Kochetkov and V. N. Shibaev, Izv. Akad. Nauk SSSR. Ser. Khim. 1169-1189 (1974).
2. O. Lüderitz, O. Westphal, A.-M. Staub and H. Nikaido, Microbial Toxins 4, 145-233 (1971).
3. P. W. Robbins and T. Uchida, Biochemistry 1, 323-335 (1962).
4. M. Fukuda, F. Egami, G. Hämmerling, O. Lüderitz, G. Bagdian, and A.-M. Staub, Eur. J. Biochem. 20, 438-441 (1971).
5. A. Wright, M. Dankert, P. W. Robbins, Proc. Nat. Acad. Sci. U.S.A. 54, 235-241 (1965).
6. C. G. Hellerqvist, B. Lindberg, J. Lönngren, and A. A. Lindberg, Acta Chem. Scand. 25, 939-944 (1971).

7. C. G. Hellerqvist, B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg, Carbohyd. Res. 8, 43-55 (1968).
8. C. G. Hellerqvist, B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg, Carbohyd. Res. 9, 237-241 (1969).
9. H. Kito and H. Nikaido, J. Bacteriol. 113, 672-679 (1973).
10. I. M. Weiner, T. Higuchi, L. Rothfield, M. Salmarsh-Andrew, M. J. Osborn, and B. L. Horecker, Proc. Nat. Acad. Sci. U.S.A. 54, 228-235 (1965).
11. C. G. Hellerqvist, B. Lindberg, A. Pilotti, and A. A. Lindberg, Carbohyd. Res. 16, 297-302 (1971).
12. C. G. Hellerqvist, B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg, Carbohyd. Res. 14, 17-26 (1970).
13. C. G. Hellerqvist, B. Lindberg, J. Lönngren, and A. A. Lindberg, Acta Chem. Scand. 25, 601-606 (1971).
14. H. J. Kopmann and K. Jann, Eur. J. Biochem. 60, 587-601 (1975).
15. B. A. Dmitriev, A. Ya. Chernyak, and N. E. Bayramova, Izv. Akad. Nauk SSSR Ser. Khim. 142-148 (1975).
16. N. K. Kochetkov, E. M. Klimov, and V. I. Torgov, Izv. Akad. Nauk SSSR. Ser. Khim. 165-167 (1976).
17. N. K. Kochetkov, B. A. Dmitriev, O. S. Chizhov, E. M. Klimov, N. N. Malysheva, V. I. Torgov, A. Ya. Chernyak, and N. E. Bayramova, Izv. Akad. Nauk SSSR. Ser. Khim. 1386-1392 (1974).
18. N. K. Kochetkov, B. A. Dmitriev, N. N. Malysheva, A. Ya. Chernyak, E. M. Klimov, N. E. Bayramova, and V. I. Torgov, Carbohyd. Res. 45, 283-290 (1975).
19. N. K. Kochetkov, B. A. Dmitriev, and A. V. Nikolaev, Izv. Akad. Nauk SSSR. Ser. Khim. 2578-2581 (1977).
20. N. K. Kochetkov, N. N. Malysheva, V. I. Torgov, and E. M. Klimov, Carbohyd. Res. 54, 269-274 (1977).
21. V. N. Shibaev, V. N. Chekunchikov, and N. K. Kochetkov, Izv. Akad. Nauk SSSR. Ser. Khim. in press.
22. D. L. MacDonald, Methods Carbohyd. Chem. 6, 389-392 (1972).
23. V. N. Shibaev, Yu. Yu. Kusov, M. F. Troitzky, and N. K. Kochetkov, Izv. Akad. Nauk SSSR. Ser. Khim. 182-184 (1974).
24. M. J. Osborn, M. A. Cynkin, G. L. Müller, and M. Singh, Methods Enzymol. 28, 583-601 (1972).
25. F. W. Hemming, MTP International Review of Science. Biochemistry, Ser. 1., Vol. 4, Butterworths, London, 1974, pp. 39-97.
26. T. Mánkowski, W. Sasak, E. Janczura, and T. Chojnacki, Arch. Biochem. Biophys. 181, 393-401 (1977).
27. H. Fukawa, M. Toyoda, T. Shimizu, and C. Murohashi, Tetrahedron Lett. 6209-6213 (1966); M. Toyoda, H. Fukawa, and T. Shimizu, J. Agr. Chem. Soc. Japan 43, 688-693 (1969).
28. G. I. Vergunova, I. S. Glukhoded, L. L. Danilov, G. I. Eliseeva, N. K. Kochetkov, M. F. Troitzky, A. I. Usov, A. S. Shashkov, and V. N. Shibaev, Bioorg. Khim. 3, 1484-1492 (1977).
29. C. D. Warren and R. W. Jeanloz, Biochemistry 11, 2565-2572 (1972).
30. V. N. Shibaev, Yu. Yu. Kusov, T. N. Druzhinina, N. A. Kalinchuk, N. K. Kochetkov, S. S. Rozhnova, and V. A. Kilesso, Bioorg. Khim. 4, 47-56 (1978).
31. C. D. Warren and R. W. Jeanloz, Biochemistry 14, 412-419 (1975).
32. C. D. Warren, Y. Konami, and R. W. Jeanloz, Carbohyd. Res. 30, 257-279 (1973).
33. C. D. Warren and R. W. Jeanloz, Carbohyd. Res. 37, 252-260 (1974).
34. H. A. Staab and K. Wendel, Lieb. Ann. 694, 86-90 (1966).
35. L. M. Kolobushkina, A. M. Krytzin, and V. L. Florent'ev, Khim. Geterozyk. Soed. 996-1000 (1973); A. M. Krytzin, S. M. Mihailov, L. I. Kolobushkina, N. S. Padjukova, and V. L. Florent'ev, Izv. Akad. Nauk SSSR. Ser. Khim. 1846-1850 (1975).
36. V. N. Shibaev, Yu. Yu. Kusov, N. A. Kalinchuk, and N. K. Kochetkov, Bioorg. Khim. 3, 120-127 (1977).
37. Yu. Yu. Kusov, N. A. Kalinchuk, and V. N. Shibaev, Abstr. IXth Int. Symp. Carbohyd. Chem. London (1978), paper G.23.
38. V. N. Shibaev, T. N. Druzhinina, G. I. Eliseeva, and N. K. Kochetkov, Bioorg. Khim. 4, 257-268 (1978).
39. V. N. Shibaev, T. N. Druzhinina, N. K. Kochetkov, Š. Kučar, and Š. Bauer, Bioorg. Khim. 4, 410-414 (1978).

40. N. K. Kochetkov and V. N. Shibaev, Advan. Carbohyd. Chem. Biochem. 28, 307-399 (1973).
41. V. N. Shibaev, Yu. Yu. Kusov, V. A. Petrenko, and N. K. Kochetkov, Izv. Akad. Nauk SSSR. Ser. Khim. 887-891 (1976).
42. V. N. Shibaev, Yu. Yu. Kusov, V. A. Petrenko, and N. K. Kochetkov, Izv. Akad. Nauk SSSR. Ser. Khim. 2588-2591 (1976).
43. V. N. Shibaev, G. I. Eliseeva, Yu. Yu. Kusov, V. A. Petrenko, S. S. Mitchenko, and N. K. Kochetkov, Izv. Akad. Nauk SSSR. Ser. Khim. 2584-2587 (1976).
44. V. N. Shibaev, Yu. Yu. Kusov, V. A. Petrenko, and T. N. Druzhinina, Bioorg. Khim. 4, 249-256 (1978).
45. V. Ginsburg, J. Biol. Chem. 241, 3750-3753 (1966).