IMMUNOCHEMICAL APPROACHES TO THE STRUCTURAL CHEMISTRY OF POLYSACCHARIDES

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ABSTRACT

Based on quantitative precipitin theory, it is possible to identify some sugar groupings in a polysaccharide molecule by immunochemical cross-reactions.

The structural features of some type-specific pneumococcal polysaccharides are shown and the reasons for their cross-reactions in different antipneumococcal sera are discussed. Verification of these conclusions was made by studying the cross-reactions of different polysaccharides having similar sugar grouping, in these sera. Hualtaco and mangle gums precipitated antibodies in different antipneumococcal sera and the reasons for this are discussed from the structural point of view. Immunochemical methods are also of great help in establishing the homogeneity of a polysaccharide.

Some limitations to the use of this method for identifying the sugar residues in a polysaccharide molecule are also indicated. One of them could be explained on the basis of stereochemical identity of some monosaccharide units, as in the case of 3-*O*-methyl-D-galactose and L-fucose. The largest dimensions of antibody combining sites are complementary to those of an oligosaccharide containing six hexopyranose units. Studies on the size and shape of this molecule will help in making the inhibition technique more widely applicable. Immunochemical methods are becoming more useful in structural polysaccharide chemistry.

Polysaccharides are the third largest group of biopolymers; they have attracted the attention of chemists for more than a century. Structural elucidations of these substances are challenging problems to organic chemists, and a major break-through was achieved with Haworth's methods of methylation. Oxidation by periodate, Barry and Smith degradation and graded hydrolysis are some of the well-known methods used to interpret the structural details of these macromolecules[1]. Spectral methods[2] have recently been utilized for characterizing the small fragments isolated from various derivatives of polysaccharides. Aspects such as size and shape have not been studied using physicochemical methods, except in a few cases such as cellulose and starch, although this problem is important in understanding the behaviour of these molecules in solution. The three-dimensional structure of monosaccharide molecules can be ascertained by x-ray crystallography.

Indeed, substantial progress has been made in the structural elucidation of polysaccharides but much remains to be understood. This is mainly due to the polyfunctional nature of the monosaccharide units in the polymer;

added to this, each sugar unit may be α or β glycosidic and pyranose or furanose and any of the hydroxyl groups may be etherified or esterified. We have been writing the structure of a polysaccharide as its repeating unit and it is not certain whether this kind of representation is the actual situation or whether, as the biosynthesis of these substances follows statistical principles, the repeating unit is a mean of all these arrangements in the macromolecule.

Polysaccharides have diverse functions in nature, and structural studies are of special interest in understanding the relation between structures and their biological roles. In the case of many bacteria, immunological specificity is assigned to capsular or *O* somatic polysaccharides. While trying to understand their serological specificities, immunochemists have developed new methods to identify some sugar groupings in them and I shall deal with this recent approach to the structural elucidation of polysaccharides.

It is desirable to give a short introduction about antigen and antibody for those who are not immunologists. If a microbial or viral agent crosses the defensive mechanism of an animal body, reactions set in with the resultant formation of new protein in the blood. These were shown to be globulin in character[3–5] and were present in the γ-globulin fraction of serum proteins; they are called antibodies. The material that stimulates antibody production is called antigen. Antibody and the corresponding antigen, which is either a whole cell or a soluble material, combine to give agglutination of the cells or precipitation, and the reactions are called the agglutinin or precipitin reaction, respectively. These two reactions involve essentially the same process.

Some forty years ago Marrack and Smith[6] considered antigens and antibodies to be multivalent with respect to each other. Now we know that antigens are multivalent and that the majority of antibodies are at least divalent. If a molecule of antigen combines with one molecule of antibody, there are still left on antigen and antibody molecules other sites that can take part in this reaction. This process goes on until large aggregates are formed and the affinity for water is lost owing to the discharge of large numbers of positive and negative charges on the macromolecules; finally, the precipitate separates out from the solution. Taking advantage of this reaction, Heidelberger and Kendall[7], for the first time, measured the amount of antibody present in an immune serum in units of weight, and an analytically pure antibody was isolated.

Out of these measurements evolved the important theory of the quantitative precipitin reaction developed by Heidelberger and Kendall in 1935[8]. The combination of antigens and antibodies is due to the presence of specific groupings of structural units in the antigen molecule and a corresponding complementary site on the antibody molecule. Hence, one of the outcomes of the quantitative precipitin reaction is that if two polysaccharides (or proteins) contain multiples of the same groupings in common, it is to be expected that each of them would precipitate the antibody raised against the other. To illustrate: after injecting *Pneumococcus* (Pn) Type I and II bacteria separately into two animals, the corresponding antisera can be obtained. If Pn Type I bacteria and its antiserum are mixed, agglutination of cells takes place but not with Pn Type II bacteria, and vice versa. This clearly indicates that there are no common specific groupings in these two antigens.

Much work has been carried out on the structure and immunological specificities of pneumococcal and other microbial polysaccharides and, in fact, these investigations initiated the subject of this report. *Pneumococcus* in its virulent form has a slimy capsule which is mainly composed of polysaccharide material. The immunological specificity of the bacteria has been shown[9] to derive from it. There are about 80 types of *Pneumococci* and each type has its characteristic polysaccharide. Since antibodies are proteins, it is convenient to consider some pneumococcal polysaccharides which

Table 1. Homologous and cross-reactions of SIII and SVIII in PnIII and PnVIII antisera

Specific polysaccharide	Amount, μg	Antibody N precipitated in 1.0 ml of PnIII antiserum H792, μg	Antibody N precipitated in 1.0 ml of PnVIII antiserum H 644, μg
SIII	50	664	
	75	702	
	100	700	
	89		289
	118		327
	177		368 (excess S)
SVIII	75		1036
	100		1128
	150		1116
	200		1110
	150	345	
	200	378	
	250	382	
	300	408	
	400	422 (excess S)	

do not contain nitrogen. One with a very simple structure is the Type III pneumococcal polysaccharide (here and elsewhere these polysaccharides are represented by S followed by a Roman numeral corresponding to the type number; for example SIII). It is composed of cellobiouronic acid which is joined to the next unit through 1→3 linkage[10, 11]. The structure is shown in *Figure 1*. A fragment of this polysaccharide having a molecular weight of 700–2000, was found to precipitate[12] the corresponding antibody in a horse serum indicating that these specific polysaccharides were multivalent.

The cross-reactions between Types III and VIII pneumococcal polysaccharides were among the earliest systems studied[13, 14] extensively and *Table 1* contains the results. The chemical reason for these cross-reactions was understood when Goebel showed[15] that SIII and SVIII contained the same aldobiouronic acid, viz., cellobiouronic acid. The fine structure of SVIII was established much later[16] and appears in *Figure 1*. The cellobiouronic acid portion responsible for the cross-reactions in this system is marked by dashed lines. That this aldobiouronic acid is responsible for immunochemical cross-reactions can be established by using cellobiouronic acid to inhibit homologous and cross-reacting systems; see further below. The fact that these two polysaccharides were linear was deduced by immuno-

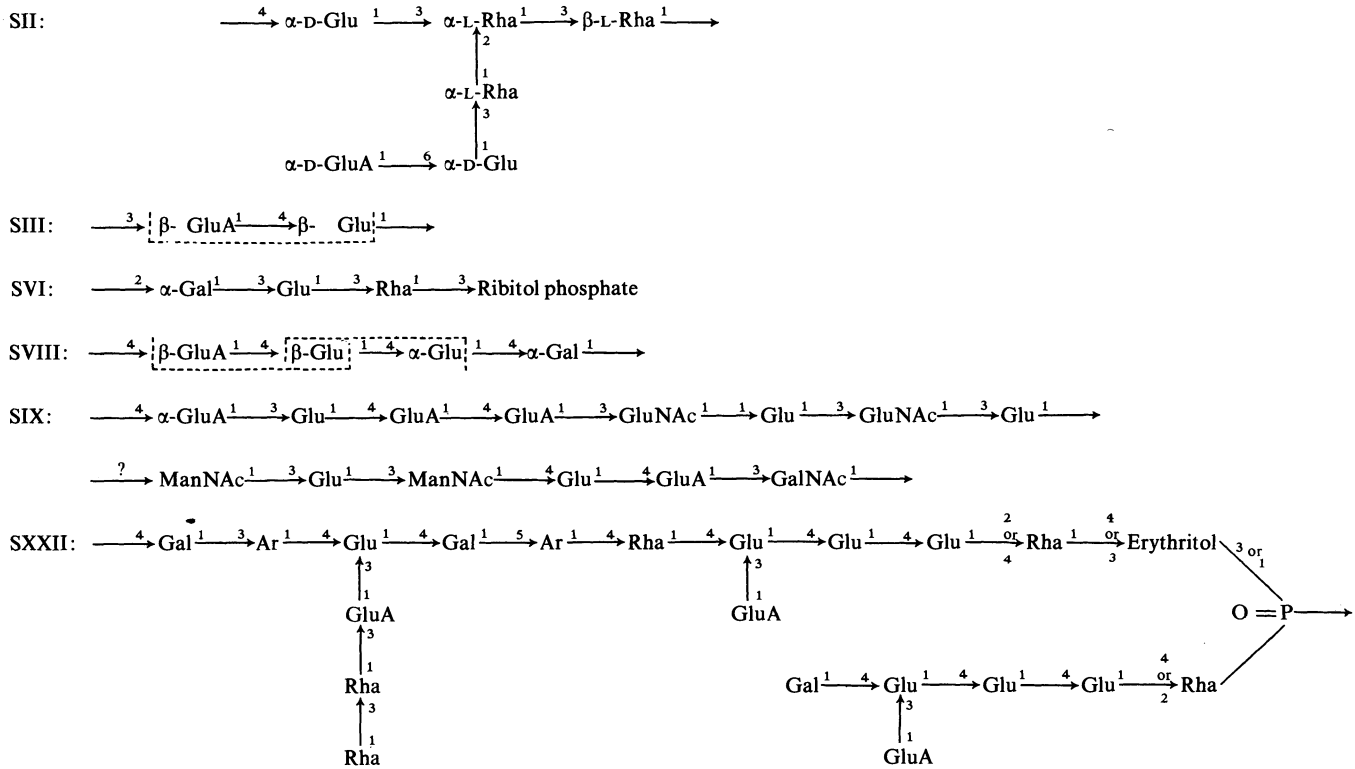


Figure 1. Structural formulae of the repeating units of pneumococcal polysaccharides (arabinose in the L-furanose form, rhamnose in the L-pyranose form and other sugars in the D-pyranose form)

chemical methods much earlier than it was established by more rigorous chemical methods.

Another part of the molecule of SVIII in *Figure 1* is marked by a dotted line and contains β-(1→4)-linked glucobiose. According to the theory of the quantitative precipitin reaction, polysaccharides containing this sugar grouping should precipitate antibody from Type VIII pneumococcal antiserum. Barley and oat glucans did cross-react[17] in this serum and subsequently this oligosaccharide, cellobiose, was isolated from them and chemically identified.

Another interesting set of cross-reactions in Pn Type II antiserum was explained on a chemical basis only when the main structural features of SII were elucidated by Butler and Stacey[18]. A revised structure was assigned[18a] to its repeating unit and is shown in *Figure 1*. The glucose units in this molecule are linked 1,4- and 1,6-. Polysaccharides containing 1,4-linked glucose units are expected to precipitate a portion of antibody from this serum; glycogen and amylopectin cross-reacted in PnII antiserum[19,20]. Some interesting observations were made during these studies: (1) amylopectin precipitated less antibody than did glycogen, and (2) removal of portions of side chains in amylopectin increased the amount of nitrogen precipitated from the antiserum. It may be possible that steric factors are playing important roles in these reactions.

Tamarind seed polysaccharide also contained 1,4-linked glucopyranose units[21,22] but the glycosidic linkage was β. This polysaccharide also showed cross-reaction in PnII antiserum. It is to be pointed out that the nature of the anomeric linkage does not seem to have much effect on the cross-reactions when the specific sugar groupings are in the interior of the polysaccharide molecule, in contrast to those at the non-reducing ends, which show marked differences, as observed by Avery *et al.*[23]. Dextrans having α-1→6 linkages were also found[24–26] to precipitate a portion of antibody nitrogen from PnII antiserum. This can be ascribed to the presence of 1,6-linked glucose units in SII and the dextran. It is also possible that the non-reducing end hexopyranose residue may fit into antibody spaces designed for glucuronic acid units. The above two examples illustrate some of the limitations of immunochemical methods for the identification of sugar groupings in a polysaccharide molecule.

It was observed[27] by Avery in 1915 that some strains of *Pneumococcus* gave weak agglutination tests with PnII antiserum and he designated them as IIA, IIB, etc. Subsequently the first two were numbered types V and VI[28], as they were responsible for many cases of pneumonia. Although the specific substances of Pn V and Pn VI differ chemically, the cross-reactions indicate that they have some common sugar groupings with SII. Barker and his associates worked on the structure of SV and Rebers and Heidelberger on SVI. *Figure 1* contains the structure of the repeating unit of SVI[29]. One can see that D-glucose and L-rhamnose are the common sugar units present in SII and SVI molecules. The glucose residues in SII are linked 1,6- and 1,4-, whereas that in SVI is 1,3-. This sugar was not involved in the specificities for cross-reactions between the PnII and PnVI systems, since glucans that precipitated antibody from anti-PnVI did not react in anti-PnII, whereas those giving a reaction in anti-PnII showed either a minor or no

reaction in PnVI antiserum. Hence, the only sugar for consideration was L-rhamnose. Group A haemolytic *Streptococcus* gave a weak reaction in PnII antibody solution and the only common sugar in these two specific substances was L-rhamnose[30]. A variant[31, 32] of the former material, containing a greater proportion of L-rhamnose, showed strong cross-reactions in PnII[33] and PnVI[34] antisera. These results show that the presence of 1,3-linked L-rhamnose residues in SII and SVI is responsible for cross-reactions between PnII and PnVI.

Table 2. Inhibition of precipitation of isolichenin in antipneumococcal Type IX sera. Total volume, 0.50 ml. H, horse; R, Rabbit

Inhibitor	μmol added	Antibody N precipitated from		Inhibition %
		H 963C, μg	R 91_2 μg	
None			35	
D-Glucose	75		3	91
	25		17	51
Cellobiose	30		20	43
None		44		
D-Glucose	100	30		32
Na D-glucuronate	29	46		0
N-Ac-D-glucosamine	45	36		18
None			39	
Methyl α-D-glucoside	31		26	33
Methyl β-D-glucoside	31		26	33
None			31	
N-Ac-D-glucosamine	45		14	55
Cellobiose	24		19	39
Maltose	23		5	84
Maltose	5		15	52
Maltose	2.5		20	36
None		32		
Cellobiose	55	29		9
Maltose	15	21		34
Maltose	5	24		25

In the SIX–anti-SIX reactions, under comparable conditions, *N*-acetyl-D-glucosamine, 45 μmol, gave 14 per cent inhibition; the other sugars scarcely inhibited.

PnIX antisera were shown to give cross-reactions with a wide variety of natural and synthetic polyglucoses and some plant gums[35–37]. From the results of investigations started by Heidelberger and Rao[38] and later continued by Das and Higginbotham[39, 40] a tentative structure was assigned to the repeating unit of SIX, which was also shown in *Figure 1*. Goodman and Kabat observed that 1→2-, 1→3- and 1→4-linked dextrans reacted in PnIX antiserum more strongly than 1→6-linked ones and that α-1→2, α-1→3 and α-1→4-linked di- and trisaccharides of glucose were potent

inhibitors. It can be seen that the proposed structure for SIX contains 1,3- and 1,4-linked glucose units but nowhere are there two consecutive glucose residues. When inhibition studies were conducted on isolichenin–PnIX antisera systems (*Table 2*), maltose showed the greatest inhibition. Another disaccharide, 3-*O*-α-D-glucuronopyranosyl-D-glucose, also showed strong inhibition in homologous and isolichenin systems and the results

Table 3. Inhibition of homologous and isolichenin cross-reacting systems in PnIX antisera by salts of acidic disaccharides. Dilutions of PnIX antisera H 623C and R 91 were used (C, absorbed with group-specific Pn- C substance). The total volume was 0.5 ml in all runs

Homologous system Sugar added	Antiserum	Time at 0°C days	Antibody N pptd μg	Inhibition, %
None	Horse	8	24	
3-*O*-α-D-glucuronopyranosyl-D-glucose (30 μmol)	Horse	8	13	46
None	Rabbit	8	23	
3-*O*-α-D-glucuronopyranosyl-D-glucose (30 μmol)	Rabbit	8	12	48
None	Rabbit	7	34	
α-4-*O*-D-glucuronopyranosyl-D-glucose (60 μmol)	Rabbit	7	30.5	10
None	Rabbit	8	32	
α-4-*O*-D-glucopyranosyl-D-glucuronic acid (76 μmol)	Rabbit	8	27	16
Cross-reacting system				
None	Horse	8	18.5	
3-*O*-α-D-glucuronopyranosyl-D-glucose (5 μmol)	Horse	8	11.5	38
None	Rabbit	8	36	
3-*O*-α-D-glucuronopyranosyl-D-glucose (5 μmol)	Rabbit	8	2	94
3-*O*-α-D-glucuronopyranosyl-D-glucose (1.25 μmol)	Rabbit	18	6	83
None	Rabbit	9	33.5	
α-4-*O*-D-glucuronopyranosyl-D-glucose (30.5 μmol)	Rabbit	9	17.5	48
α-4-*O*-D-glucopyranosyl-D-glucuronic acid (38 μmol)	Rabbit	9	11	67

are indicated in *Table 3*. These results strongly suggest the possibility that a second glucose residue in the disaccharide molecule is occupying a place in the antibody combining site, complementary to glucuronic acid. Such observations were made earlier in the case of glycogen–PnII antiserum[41] and oat glucan–PnVIII antiserum[42] systems. This is another limitation of the use of immunochemical methods for the structural elucidation of polysaccharides.

Many polyglucoses and plant gums cross-reacted with PnXXII antiserum also. We worked on the structure of SXXII, the repeating unit of which is tentatively[43] set out in *Figure 1*. This polysaccharide contains L-arabinose

as a constituent, and this sugar is not encountered in any other pneumococcal polysaccharide whose structure is known. There are some 1→4-linked glucobiose groupings in this molecule,and this portion of the polysaccharide may be responsible for cross-reactions with polyglucoses. We are now continuing our investigations to interpret the fine structural details of this polysaccharide.

The reasons for the cross-reactivity of polysaccharides in an immune serum have been examined. Sometimes the results may be misleading. A positive reaction definitely indicates the presence of multiples of common specific groupings in the polysaccharide under examination and the corresponding specific material against which the antiserum has been raised. But a negative reaction should be examined carefully, as the particular antiserum used may not contain cross-reacting antibodies. In such cases tests should be conducted in antisera obtained from different animals and then conclusions drawn. Immunologically specific polysaccharides from microorganisms were found to have many structural similarities to those of plant gums. Heidelberger tested the cross-reactivity of many plant gums in various pneumococcal antisera qualitatively and the results were published[35]. Subsequently many polysaccharides from microbiological and plant sources were added to the record. A few examples follow. It was found by Heidelberger and Rao[44] that hualtaco gum from the plant *Loxopterygyum huasango* precipitated about 75 per cent of antibody nitrogen from PnXXIII antiserum and showed a strong cross-reaction in PnII antiserum, as did gum arabic; the results of cross-reactions are shown in *Table 4*. The hualtaco gum contains D-galactose, L-arabinose, L-rhamnose and D-glucuronic acid. The aldobiouronic acid was characterized as 6-*O*-(β-D-glucuronopyranosyl)-D-galactose. Precipitation in PnII antiserum was ascribed to the presence of non-reducing end units of D-glucuronic acid in the molecule. Lesser amounts of degraded gum could bring about maximum precipitation, indicating that some of the labile linked sugar residues were removed during degradation of the gum, thus making more uronic acid residues available for reaction. With PnXXIII antiserum almost the same amount of intact and degraded gum brought about maximum precipitation.

Precipitation of PnXXIII antibody was due to other sugar groupings. What we know about SXXIII is that it contains D-glucose, D-galactose, L-rhamnose, phosphorus and no uronic acid[45]. As the common sugars in these two polysaccharides were D-galactose and L-rhamnose, the heavy cross-reaction was thought to be due to the presence of similarly linked common sugars or a combination of them. With these results in view Samajpathi and Rao set out to elucidate the structure of hualtaco gum. The structures illustrated in *Figure 2* are those of degraded gum[46], the whole gum (unpublished data) and gum arabic[47]. If a comparison is made between the structures of hualtaco and arabic gums, some common structural features can be identified. D-galactose and aldobiouronic acid residues at the non-reducing ends and 1,3,6-linked D-galactose units at the branch points are present in both gums. A word of explanation is necessary about rhamnose units in gum arabic. Earlier reports showed substantial amounts of this sugar, which was present as non-reducing end units, and the results given in our paper[44] were obtained using some of the earlier samples. But the sample

used by Anderson *et al.*[47] was shown to contain only 0.4 per cent of rhamnose and, as one can see in the structure, no place was given to it. Thus it is desirable to test this sample for serological reactivity. In hualtaco gum L-rhamnose units are present in some of the non-reducing ends. Studies[48]

Table 4. Cross-reactions of hualtaco gum, its derivatives, and certain other polysaccharides in antipneumococcal sera of types I, II, XXII and XXIII

Polysaccharide	Amount (mg)	Antibody nitrogen precipitated at 0°, calculated to 1.0 ml from serum, type and No.					
		I 884 C[a] µg	I 1057 C[a] µg	I 1057FC[b] µg	II 513 µg	XXII 566[c] µg	XXIII 912 µg
Homologous	At max. pptn	980	1024	*ca.* 1000	3600	374	275
Hualtaco	0.15					35	
Fr. I	0.20			33			192[d]
	0.30	33	19	30		36	200[d]
	0.60	36	20	36	29[e]		
	10.00				256		
	25.00				319		
Degraded	0.15					30	
Fr. I	0.20						175
	0.30	15		44	369	28	185
	0.50				487		
	0.60	16		31			
	1.00				483		
Oxidized	0.1	0					
Fr. I	0.25	0			16		
Oxidized	0.5			+ + +	523[f]		
Degraded	1.0				561[f]		
Fr. I							
Gum arabic	0.6						234
	2.0						229
	100.0				1197[g]		
Degraded	0.2						197[h]
gum arabic	0.3						209[h]
	0.5				626[g]		

[a] Absorbed with pneumococcal C-substance; Sera II 513 and XXIII 912 contained only traces of anti-C. [b] Felton solution from I 1057, absorbed with C-substance. Fraction II precipitated the same amount of antibody from this as did Fraction 1 but required larger amounts. [c] Absorbed with C-substance and partially with glycogen. [d] Supernatants plus SXXIII at the 13 µg level gave 51 µg N. Oxidized Fraction I gave little or no precipitate in anti-PnXXIII. [e] From precipitin curve. [f] Precipitates formed very slowly. Supernatants of tubes containing I mg were opalescent. [g] From earlier studies. After absorption with degraded gum arabic II 513 gave 11 µg N with degraded hualtaco gum; after absorption with ketha gum (earlier studies), 64 µg N. [h] Supernatants plus hualtaco. Fraction I, at the 160 µg level, gave 9 µg N.

on the cross-reactions of different systems showed the presence of non-reducing end units of L-rhamnose. It is possible that the small amount (0.4 per cent) of this sugar detected in the sample obtained by Anderson *et al.*[47], is present as non-reducing ends.

The other substance for consideration here is mangle gum (*Rhizophora mangle* L), which contains D-galactose, L-rhamnose, L-arabinose, D-galac-

turonic acid, D-glucuronic acid and its 4-*O*-methyl derivative; the total acid sugar content is 46 per cent. The results[49] of cross-reactions in various pneumococcal antisera are shown in *Table 5*. The structures of degraded and whole gum[50] (unpublished data) are shown in *Figure 3*. From these results it is possible to explain the cross-reactions of mangle gum. A strong reaction in PnI antisera was due to the presence of D-galacturonic acid residues at non-reducing ends, as the same antibody that reacted with *Khaya senegalensis* was involved. The massive reaction in PnII antiserum was ascribed to non-reducing D-glucuronic acid residues or their 4-*O*-methyl

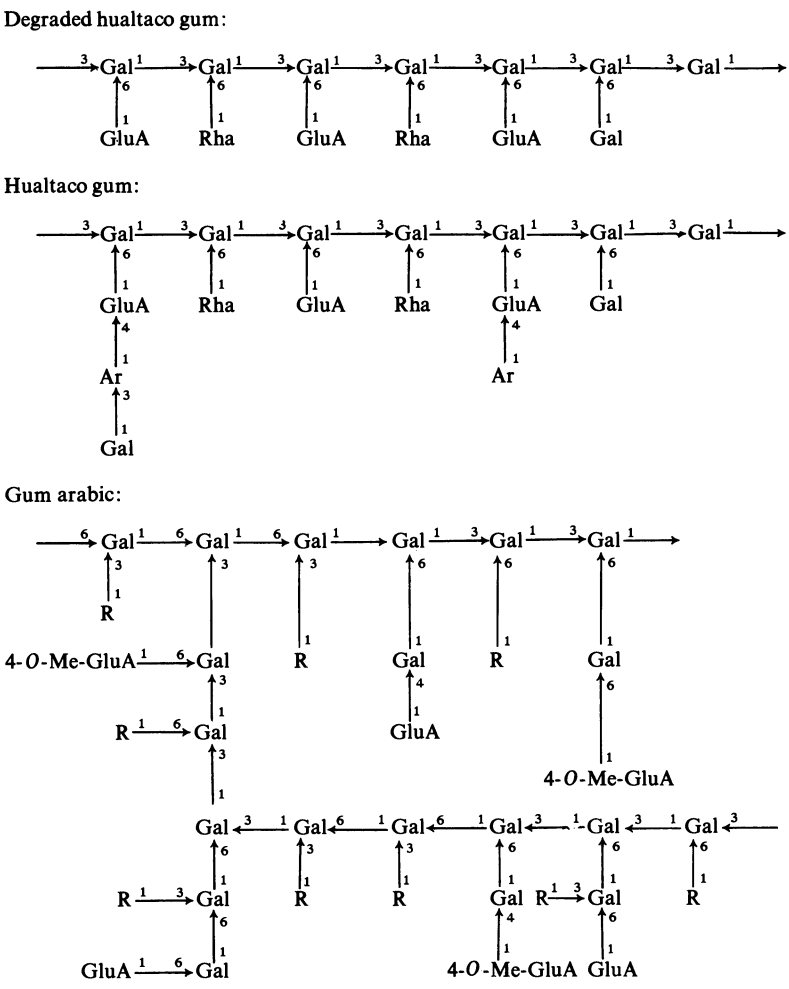
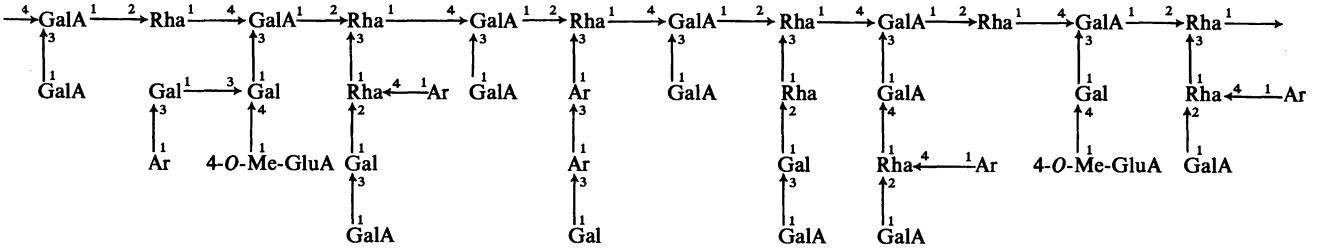


Figure 2. Structural formulae of the repeating units of degraded and whole hualtaco gum and gum arabic (arabinose in the L-furanose form, rhamnose in the L-pyranose form and other sugars in the D-pyranose form). R represents L-arabinofuranose or L-arabinopyranose containing side chains. Some of these side chains are at least six units long

Degraded mangle gum:



Mangle gum:

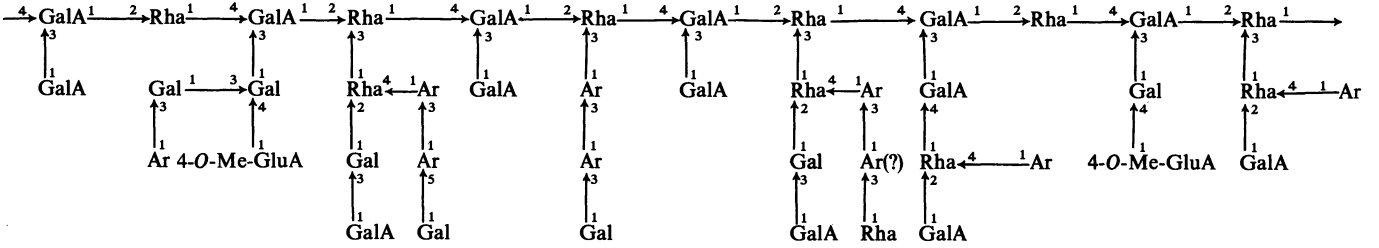


Figure 3. Structural formulae of the repeating units of degraded and whole mangle gum (arabinose in the L-furanose form, rhamnose in the L-pyranose form and other sugars in the D-pyranose form)

Table 5. Cross-reactions of mangle tree gum and derivatives in antipneumoccal horse sera. Micrograms antibody nitrogen precipitated at 0°, calculated to 1.0 ml antiserum

Amount of polysaccharide μg	Antipneumococcal horse serum, type						
Homologous at max.	I 704C[a] 2200	I 884C[a] 970	I 1057C[a] 1020	II 513[b] 3600	VI 681C[a] 724	XIV 635C[a] 990	XXIII 912[b] 275
Mangle							
50			38		50[g]	45	
100		38	58, 74[e]		65[g]	69	55[h]
200		52, 65[d]	52, 84[e]		51[g]	56	66[h]
400		53, 60[d]					80[h]
500	205						
800							73[h]
1000	228						
1500				1200			
3000				1200			
Degraded gum							
50						63	
100			94[f]			59	21
200	212[c]		88[f]				22
400	236[c]						
1000				1320[i]			
2000				1380			
Periodate oxd gum							
100							6
200							3
Mangle, Fr. A_1							
250				850			
400	182						
500	176			1225			
1000				1115			

[a] Absorbed with pneumococcal C-substance. [b] These sera contained negligible anti-C. [c] Supernatants plus *Khaya senegalensis* at the 1 mg level gave 66 μg N; intact serum gave 255. [d] Supernatants plus *Khaya senegalensis* at the 1 mg level gave 29 μg N; intact serum gave 52 μg N. [e] Supernatants plus *Khaya senegalensis* at the 1 mg level gave 42 μg N; intact serum gave 188 μg N. [f] Supernatants plus *Khaya senegalensis* at the 1 mg level gave 8 μg N. [g] Supernatants plus dextran 1355-S gave 32 μg N, as in intact serum; supernatants from this plus guar gave 44 μg N; intact serum gave 106 μg N. [h] Supernatants plus *E. coli* K85 gave 9 μg N; intact serum gave 20 μg N. [i] Serum II 513 absorbed with degraded gum arabic (626 μg N pptd during earlier studies) gave 840 μg N with degraded mangle gum at the 1 mg level. Serum II 513 gave no ppts with varying amounts of oxidized–reduced mangle gum.

derivatives as PnII antisera do not distinguish between these two sugars in a polysaccharide molecule[51–53]. In fact, in our cooperative research Heidelberger detected first the 4-*O*-methyl derivative by serological methods followed later by the chemical investigations. In the non-reducing ends of the polysaccharide the number of D-galactose units is less than the number of other sugar residues and, as expected, the reactions in PnVI[54] and XIV[55] antisera, that were ascribed to these sugar units in a molecule, were not strong. A reaction in PnXXIII was ascribed to the presence of L-rhamnose units at non-reducing ends and this was further supported by the fact that

the mangle gum and the capsular polysaccharide of *Escherichia coli* K85[56], in which L-rhamnose occurred in the same form, precipitated the same fraction of antibody. A weak reaction in this serum by periodate-oxidized material was probably due to L-rhamnose units present in the interior of the polysaccharide molecule.

One can multiply the number of examples in which the sugar groupings in a polysaccharide molecule are identified from the study of serological cross-reactions. The capsular polysaccharides of different types of *Klebsiella* and *Rhizobia* have some common structural features with those of pneumococcal substances[57]. One interesting structural unit in them is pyruvic acid, which has been shown to have immunological specificity. *Klebsiella* K^3 material containing 0.4 per cent pyruvic acid cross-reacted in anti-PnXXVII, and K32 with 8.4 per cent of this acid precipitated antibody from anti-PnIV. This led to the prediction of cross-reactions between PnIV and PnXXVII; these were due to the presence of pyruvic acid in SIV and SXXVII. Rhizobial polysaccharides, on depyruvilation, showed much greater cross-reactivity and occasionally different reactivity in anti-Pn sera, except with anti-PnXXVII, in which precipitation is virtually abolished. Considerable work has also been carried out to elucidate the cross-reactions, in both directions, of specific substances and the corresponding antisera of *Salmonella* and *Pneumococcus*.

The power of immunochemical methods can be illustrated by another example. Ketha gum from the tree *Feronia elephantum* was reported[58] to contain D-glucuronic acid besides other sugars. Serological cross-reactions in PnII antiserum[35] confirmed the presence of this sugar, but a reaction was observed in PnI antiserum which was due to the presence of D-galacturonic acid residues in the molecule[59, 60]. Later investigations by Heidelberger *et al.* revealed[61] that it contained D-galacturonic acid in greater proportion among the two acid sugars, while the other was present as the 4-*O*-methyl derivative and not as unsubstituted sugar.

It has been a long-standing problem for researchers working on natural polymers, and especially the polysaccharides, to isolate a pure material and test its homogeneity. Various criteria, both chemical and physical, were applied to determine the homogeneity of the isolated materials. One of the recent additions is the use of immunochemical techniques. If a polysaccharide forms a precipitate in an immune serum, the precipitate can be separated and the polysaccharide in it is isolated by dissociating the complex. If the ratio of different sugars in the polysaccharide used is the same as that obtained from the precipitate, the material may be considered to be homogeneous though this is not essentially true in all cases. The other method is to determine the ratio of the amounts of antibody and the polysaccharide in immune precipitates obtained by using different fractions of a polymer and a purified antibody solution. Identical ratios may indicate[62] the homogeneity of the material isolated.

Enough examples have been given to illustrate the usefulness of immunochemical techniques in identifying some sugar residues in a polysaccharide. One must be cautious in interpreting the results of serological cross-reactions, since there are some limitations to this method, as already indicated. In some cases it has been observed[34] that non-reducing end units

of D-galactose react as 1,2-linked D-galactose residues in anti-PnVI. In SVI the D-galactose residues are 1,2-linked, leaving positions 3, 4 and 6 free. The complementary sites on antibody molecules, directed against the latter sugar unit, may be accommodating the non-reducing D-galactose units. When such unexpected reactions are observed, other aspects such as stereochemistry of the monosaccharide units should also be considered. A 3-*O*-methyl-D-galactose molecule has the same spatial disposition of different groups as those of L-fucose when one of them is rotated on a proper axis of the molecule and superimposed on the other, whereas D- and L-galactose or fucose molecules themselves do not have similarity in profiles. In these cases replacement of a hydrophilic hydroxyl group with a hydrophobic $-CH_3$ or $-OCH_3$ group showed a marked effect in having a lower tendency to form hydrogen bonding with antibody combining sites[63]. In such situations one monomer unit in a polysaccharide molecule may behave like another when both have the same kind of spatial disposition of remaining hydrophilic groups, resulting in erroneous conclusions.

During the studies on cross-reactions of polysaccharides it is also necessary to use inhibition reactions to find out whether the same fraction of antibody having the complementary sites for a portion of a polysaccharide is involved, or if a different one is giving the precipitin reaction. Then the dimensions of the combining site on the antibody molecule play important roles. In the case of the 1→6-linked dextran–human antidextran system it has been observed[64] that a hexasaccharide, viz. isomaltohexaose, gave maximum inhibition of the homologous reaction. The immunodominant sugar of this hexasaccharide is the one at the non-reducing end and the subsequent sugar units have lesser inhibiting power[63]. Thus the greatest dimensions of antibody combining sites are those complementary to this hexasaccharide. From these observations one has to ascertain the exact contributions of different sugars to precipitation in cross-reacting systems; more difficulty is encountered when sugar units in the interior of a polysaccharide molecule are involved in the specificities. As we have already seen, the non-reducing ends containing neutral or acid sugars showed well-defined specificities in these reactions.

We know that the antibody molecules are heterogeneous in the sense that the dimensions of the combining sites vary. There is no information about the smallest size of these complementary sites and so it is difficult to say, in such situations, whether one sugar molecule alone is occupying the complementary site without regard to the glycosidic linkage or if two or more sugars are involved and to what extent. If the common sugar groupings in a cross-reacting system are at the two terminal positions and if the antibody combining site is big enough, it is not known what are the effects of different sugar units that occupy intermediate positions. What amount of cross-precipitation could occur when non-reducing ends in a cross-reacting system are not the same and at the same time do not hinder the entrance of the other sugars of the specific groupings into the antibody combining sites complementary to them, is not known.

If there is a change in structures of polysaccharides, its effect on cross-reactions is little known. It has been observed in some cases that a 1→2-linked diglucose molecule showed some inhibition of the system involving specificities for 1→4-linked glucobiose groupings in the interior of the poly-

saccharide molecule. Such cases are even more complicated where hetero-polysaccharide systems are studied. Solutions to these problems will lead to answers to many structural questions.

Some data are available[65, 66] on inhibition studies of the SIII–anti-PnIII system. In this case, also, it was observed that a hexasaccharide brought about maximum inhibition. From these results and those on the studies of the dextran–antidextran system it may be concluded that the largest dimensions of an antibody combining site correspond to those of a hexasaccharide molecule. But isomaltohexaose is neutral and α-1→6-linked, whereas the hexasaccharide obtained from SIII contains alternate acid sugars joined through β-1→4-linkages. It is expected that the latter one is a more extended molecule owing to mutual repulsion of charges on —COOH groups. It is interesting to find out whether the differences in linkages and their anomeric nature have brought about comparable sizes among these oligosaccharides. Besides, we do not know how these molecular species exist in solution and especially in an environment consisting of salts and other macromolecules from immune serum, in which these reactions are conducted. Unfortunately, we do not have suitable physical methods for determining the size and shape of these oligosaccharides in solution. It is for the carbohydrate chemists to discover a solution to these problems and to throw light on the dimensions of the combining sites on an antibody molecule.

We have reviewed some of the contributions of immunochemistry to the elucidation of the structure of polysaccharides, with their limitations. Obviously much work is needed to make this method more exact. In spite of these drawbacks, it is one of the most powerful methods for structural work on this group of biopolymers. As Professor Heidelberger has often said it is becoming more essential for a well-equipped laboratory working on structural polysaccharide chemistry to keep a well-selected library of antisera for short-cuts and guidance. It is also necessary to prepare different antisera having specificities for different sugars in a polysaccharide. Immunochemical techniques will surely have increasing applications in this branch of chemistry.

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