IMMUNOCHEMISTRY OF PNEUMOCOCCAL TYPES II, V, AND VI.

II. Inhibition Tests in the Type VI Precipitating System

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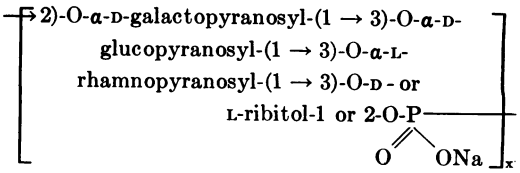
Received for publication June 19, 1961

ABSTRACT

Rebers, Paul A. (Rutgers University, New Brunswick, N. J.), Esther Hurwitz, and Michael Heidelberger. Immunochemistry of pneumococcal types II, V, and VI. II. Inhibition tests in the type VI precipitating system. J. Bacteriol. **82:** 920–926. 1961.—As in other immune systems involving polysaccharides, rabbit antibodies but not those engendered in the horse were found sensitive to degradation of type VI pneumococcal (Pn) polysaccharide (SVI), and were readily inhibited by fragments of SVI. Large amounts, 30 to 111 μmoles, of most sugars gave up to 15% inhibition, while sugar and polyol phosphates inhibited as much as 25%, with little relation to their presence or absence in SVI. The phosphate-free repeating unit of SVI was a good inhibitor, its phosphate monoester was better, and the "trimer" still better. The "trimer" precipitated most of the antibodies from horse anti-Pn VI.

Although inhibition of precipitation of SVI anti-Pn horse sera could not be demonstrated with fragments of SVI, cross-reactions of antibodies in the horse sera could be inhibited. Precipitation of SII was inhibited by low concentrations of L-rhamnose, while even high concentrations of the other sugar components of SII and SVI were ineffective. Precipitation by guar gum was inhibited by galactose and α- and β-methylgalactopyranosides, also by rhamnose, although guar gum does not contain this sugar, while SVI, the antigenic determinant, does.

The present paper deals with studies of inhibition of the homologous and cross-precipitations of type VI pneumococcal (Pn) polysaccharide (SVI) and related gums with type VI antipneumococcal horse and rabbit sera. SVI is a linear polymer composed of multiples of the repeating unit,



Rebers and Heidelberger (1959, 1961). Insight into the influence of its several components on its immunological specificity should add to knowledge in this field.

MATERIALS AND METHODS

The antisera were supplied by the Division of Laboratories and Research, Department of Health, State of New York, and the Division of Laboratories, New York City Department of Health, through the kindness of Jessie L. Hendry and Annabel Walter, respectively. Type VI pneumococcal polysaccharide was prepared by E. R. Squibb and Sons, New Brunswick, N. J. (supplied by T. D. Gerlough), and further purified (Rebers and Heidelberger, 1959). Okra mucilage (Whistler and Conrad, 1954*a*, *b*) was kindly furnished by R. L. Whistler.

Many of the inhibitors used were commercial preparations but all were analyzed for purity and most recrystallized. Quantitative precipitin estimations were carried out according to Heidelberger and Kendall (1935*a*,*b*,*c*; cf. also Kabat and Mayer, 1961). Homologous precipitin reactions were allowed to stand at 0 C for 48 hr and cross-precipitations for 12 to 15 days (Heidelberger, 1955; Heidelberger, Aisenberg, and Hassid, 1954), and, after centrifugation at 0 C, were drained in a cold box (Heidelberger and Rebers, 1958). The precipitates, after washing twice at 0 C with 0.15 M NaCl, were analyzed according to Markham (1942). For studies of inhibition of the homologous reaction in Type VI antipneumococcal rabbit serum, pool No. 14

from the N. Y. State Department of Health was diluted so that 0.20 ml contained 91 μg antibody N, of which 68 to 75 μg was precipitated by the 11 μg SVI added (antibody excess). Inhibitor solution (0.1 ml) was mixed with 0.20 ml serum, allowed to stand at 0 C for 24 hr, and 0.10 ml antigen solution added. After 48 hr at 0 C, the precipitates were centrifuged, washed, and analyzed as above.

The concentrations of the variously alkali-degraded SVI preparations were determined by analysis for phosphorus (Dryer, Tammes, and Roth, 1957).

RESULTS AND DISCUSSION

Inhibition of the homologous reaction by substances of low molecular weight. Studies by numerous workers have shown that substances of low molecular weight may specifically inhibit precipitation of antigen by antibody. If a carbohydrate antigen is branched, nonreducing terminal sugar units appear to be the most important determinants (Goebel, Avery, and Babers, 1934; Landsteiner, 1945; Goodman and Kabat, 1960*a*,*b*). Although some inhibition was observed in our system with large amounts of monosaccharides, their glycosides, and their phosphate esters (Table 1), the effect appeared to be independent of their presence or absence in SVI.

Inhibition of the homologous reaction with fragments of SVI. Since appropriate oligosaccharides are usually better specific inhibitors of the precipitin reaction than monosaccharides (Kabat, 1954), it is not surprising that the phosphate-free repeating unit of SVI, a galactoglucorhamnoribitol (Rebers and Heidelberger, 1961), is a more efficient inhibitor than ribitol, ribitol-PO_4, or any of the other component sugars listed in Table 1 (see Table 2 and Fig. 1).

Complete alkaline hydrolysis of SVI produces the monomeric repeating unit of SVI, a galactoglucorhamnoribitol phosphate (Rebers and Heidelberger, 1959). Although it differs from the preceding derivative only by the presence of a phosphate monoester group, it is four to five times more potent as an inhibitor (Table 2, Fig. 1).

In accordance with a suggestion made by Arne Tiselius, an attempt was made to assess the immunological significance of the phosphate group. The concentration of salt was increased from 0.9 to 11% to minimize the charge on the phosphate group by a strong ionic atmosphere. A portion of the experiments in the first section of Table 2 was accordingly repeated in 11% NaCl. The results are summarized in Section B, Table 2. In 11% NaCl solution the inhibitory effect of both phosphate-containing and phosphate-free repeating units was about the same, whereas the phosphate derivative was much more potent in 0.9% NaCl. If the negative charge on the phosphate derivative is attracted by a complementary

TABLE 1. *Inhibition of the homologous reaction between S VI and rabbit anti-Pn VI by substances of low molecular weight*

Inhibitor	μMoles added	Antibody N pptd.	Inhibition
		μg	%
Control		72*	
Control		68†	
Control		73*	
Raffinose	36	71*	3
D-Mannose	111	66*	8
D-Mannose	333	60†	12
D-Glucose	111	68*	5.5
D-Galactose	111	62*	14
L-Rhamnose	122	63*	12.5
D-Ribose	133	71*	1
D-Mannose-6-PO_4-Na_2	36	56*	23
D-Glucose-6-PO_4-Na_2	42	59*	19
D-Ribose-5-PO_4-Na_2	48	57*	21
D-Galactose-6-PO_4-Na_2	35	59*	19
3-Phosphoglyceric acid-Na_2	37	60*	18
D-Ribitol-5-PO_4-Na_2	39	55*	25
Polyglycerol-PO_4‡	10	60†	12
α-Glycerol-PO_4-Na_2	72	62†	9
β-Glycerol-PO_4-Na_2	79	61†	10
α-Methyl-D-galactopyranoside	88	56*	23
	264	56†	18
β-Methyl-D-galactopyranoside	95	62*	15
	285	59†	13
α-Methyl-D-glucopyranoside	95	67*	8
α-Methyl-D-mannopyranoside	95	64*	12

* Total volume of reaction mixture, 0.40 ml.
†Total volume, 0.60 ml.
‡Synthesized by A. M. Michelson, supplied by M. McCarty, used as the calcium salt, degree of polymerization, ca. 6 (McCarty, 1959).; 10 mg of this inhibitor was used instead of 10 μmoles.

TABLE 2. *Inhibition of precipitation in type VI antipneumococcal rabbit serum by fragments of S VI. A, 0.9% NaCl; B, 11% NaCl*

	Phosphate-free repeating unit*			Repeating unit phosphate†			"Trimer"‡		
	Amt. added	Antibody N pptd.	Inhibn.	Amt. added	Antibody N pptd.	Inhibn.	Amt. added	Antibody N pptd.	Inhibn.
	*μmoles**	*μg*	%	*μmoles†*	*μg*	%	*μmoles‡*	*μg*	%
A.	0	75		A. 0	74		A. 0	73	
	5	34	55	0.13	51	31	0.05	30	59
	10	27	64	0.70	44	41	0.08	21	71
	0	74		0	73		0.17	11	85
	20	28	62	1.8	28	61			
	0	73		3.7	20	73			
	33	9	88	7.4	11	85			
				14.7	2	97			
B.	0	54		0	54				
	0.7	8	82	0.35	21	61			
	1.8	6	89	0	49				
	5.3	0	100	0.7	13	74			

* Mol. wt. galactoglucorhamnoribitol·2 H_2O, 658.

† Mol. wt. galactoglucorhamnoribitol phosphate, disodium salt, 746.

‡ Calculated average mol. wt., 2100.

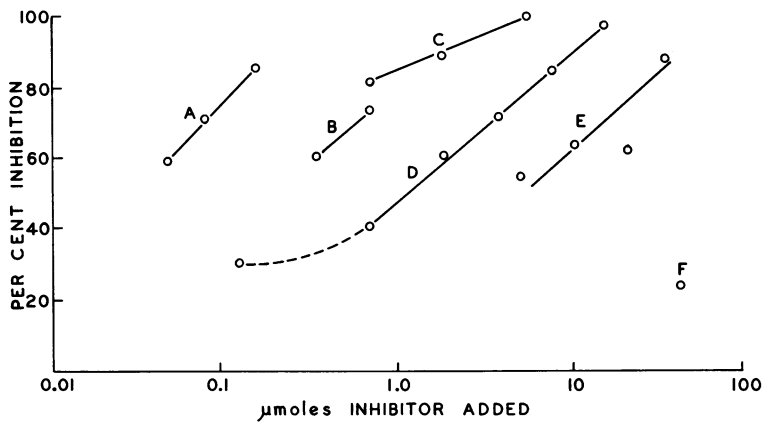


FIG. 1. *Inhibition of precipitation between type VI antipneumococcal rabbit serum and S VI by fragments of S VI. (A) Partly degraded S VI, "trimer", 0.9% NaCl; (B) Repeating unit phosphate, "monomer", 11% NaCl; (C) Phosphate-free repeating unit, 11% NaCl; (D) Repeating unit phosphate, 0.9% NaCl; (E) Phosphate-free repeating unit, 0.9% NaCl; (F)* D*-Ribitol-5-phosphate, 0.9% NaCl.*

positive charge on the antibody, then the SVI anti-SVI reaction should itself be reduced in 11% saline solution. The amount of antibody nitrogen actually precipitated from rabbit serum under these conditions was only 66 to 77% of that thrown down in 0.9% NaCl. For comparison, a neutral polysaccharide-antibody system was used as control. The specific polysaccharide of the type XIV pneumococcus, SXIV (Barker et al., 1958; Heidelberger, 1960; Barker, Keith, and Stacey, 1961), is a neutral substance which contains D-glucose, D-galactose, and N-acetyl-D-glucosamine. At equivalence, SXIV precipitated 183 μg antibody nitrogen from 0.20 ml rabbit antiserum in a final volume of 0.40 ml in 0.9% NaCl, whereas 161 μg antibody nitrogen was precipitated in 11% NaCl. Previous studies on the precipitation of type III pneumococcal polysaccharide, SIII, by antibody in the presence of strong salt (Heidelberger, Kendall, and Teorell,

1936), and on the dissociation of specific precipitates (Heidelberger and Kendall, 1936; Heidelberger and Kabat, 1938), had shown that in the case of SI, SII, and SIII the combining ratios of antibody to antigen were decreased by strong salt. Since SI, SII, and SIII are also charged polysaccharides like SVI, the shift in equilibrium previously reported for these systems was also explained by a similar mechanism involving ionic charges. As might have been anticipated, the type XIV system, in which there are no charged groups on the antigen, was much less affected by strong salt solutions.

Although hydrolysis of SVI to the monomeric repeating unit occurs with 0.02 N $Ba(OH)_2$ during 3 days at room temperature, partial breakdown into larger fragments results after shorter exposures to alkali. After 1½ hr, a back-titration showed that only 2 to 4% of the phosphate diester linkages had been split, while hydrolysis increased to 7 to 9% after 5¼ hr. Even the shorter exposure to alkali decreased the capacity of SVI to precipitate rabbit antibodies by 20%, while after 5¼ hr it was decreased by 75% (Table 3). Maximum precipitation was less in both instances and occurred with less antigen than with intact SVI, recalling the behavior of acid-degraded clinical dextran with anti-dextran serum (Kabat and Berg, 1953). In contrast to rabbit serum, horse anti-Pn VI precipitated as much antibody with both fractions of partially hydrolyzed SVI as it did with the original SVI. Similar differences between horse and rabbit antibodies were noted with the degradation products of SI, SII, and SIII (Pappenheimer and Enders, 1933; Heidelberger and Kendall, 1933; Heidelberger, Kendall, and Scherp, 1936). Although the completely alkali-hydrolyzed SVI, the monomeric repeating unit, failed to precipitate horse antibodies, it also failed to inhibit the reaction of SVI in horse anti-Pn VI.

TABLE 3. *Precipitation of anti-Pn VI rabbit serum by partially alkali-degraded S VI*

μg Antigen used	μg N pptd. by		
	S VI	Alkali-degradation 1½ hr	Alkali-degradation 5½ hr
4	37	29	11
7	58	46	13
11	72	57	18
21	88	46	6
38	91	28	3
42	90		
132	47*		

* From columns 1 and 2, an excess of 94 μg S VI resulted in 48% inhibition. By extrapolation from Fig. 1, 0.03 μmole or 63 μg of "trimer" would be required for equivalent inhibition.

TABLE 4. *Inhibition of the cross-reaction between S II and type VI anti-Pn horse serum*

Inhibitor	μMoles added	Antibody N precipitated	Inhibition
		μg	%
Control		48	
D-Glucuronic acid*	16	47	2
D-Glucuronic acid*	2	47	2
D-Galactose	111	46	4
D-Glucose	111	49	0
L-Rhamnose	11	0	100
L-Rhamnose	2.2	13	73
L-Rhamnose	1.1	25	48
α-Methyl-L-rhamnopyranoside	1.1	3	94

* An aqueous solution of glucurone was made faintly alkaline to phenol red with solid $NaHCO_3$ until the color was stable overnight. On long standing in the cold the pH was 5.7. One-tenth ml of each inhibitor solution (the others in 0.9% saline) and 0.10 ml saline were added to 0.50 ml C-absorbed horse serum 771. After 30 min, 0.10 ml S II, 250 μg/ml, was added, an amount calculated to leave a slight antibody excess. Analyses were completed after 13 days at 0 C.

A larger sample of SVI was partially hydrolyzed as follows: 100 mg of SVI in 12 ml of H_2O was treated with 0.7 ml of 0.34 N NaOH for 4½ hr at 25 C, neutralized to pH 6.5 with HCl, and dialyzed against 300 ml of distilled H_2O at 4 C. After five daily changes, the dialyzed fractions were separately concentrated in vacuo, aliquots mixed with an equal volume of double strength saline, and tested for activity against both horse and rabbit anti-Pn VI sera. All fractions, as well as the solution inside the bag, precipitated the horse serum. The first three fractions gave no precipitate with rabbit anti-Pn VI at 0 C, but the fourth and fifth showed broad diffuse bands when tested on Ouchterlony plates at 22 C. The first fraction, analyzed with intestinal alkaline phosphatase, which removed phosphate from the completely alkali-hydrolyzed SVI

(Rebers and Heidelberger, 1961) contained 36% of the total phosphate as monoester; hence, the average molecular weight was about three times that of the monomer, or 2,100. This fraction was about 36 times as potent an inhibitor of the homologous reaction in rabbit serum as was the monomer (Table 2 and Fig. 1). Since this material failed to precipitate rabbit anti-Pn VI, a test was made of whether or not the rabbit antibodies would inhibit precipitation of the "trimer" with horse antibodies. No such effect was shown.

A comparison of the "trimer" with SVI itself as inhibitor of the homologous reaction indicates that the trimer is more potent (Table 3). Efficiency of inhibition is therefore in the decreasing order: "trimer," SVI, phosphate-containing repeating unit, phosphate-free repeating unit. Apparently the length of the polymeric chain determines whether the substance is solely an inhibitor or also a precipitant in an antiserum (cf. Heidelberger and Kendall, 1933). If the size of the combining site of the antibody can be judged from the size of the most effective inhibitor (Kabat, 1954, 1960) it would appear that the size of the combining site in rabbit anti-Pn VI serum corresponds to more than one, and perhaps three, of the repeating units of SVI.

Inhibition of the cross-reactions between SII, guar and okra gums, and Type VI anti-Pn horse sera. Since the cross-precipitation of SII in type VI anti-Pn sera had been attributed to the presence of multiple groupings of 1,3-linked L-rhamnose in the specific polysaccharides of each type, it was of interest to determine the inhibiting effect of the component sugars of SII and SVI on this cross-reaction, which was most marked in serum 771 (Heidelberger and Rebers, 1960). It is apparent from Table 4 that L-rhamnose was a strong inhibitor, whereas the other sugars present in SII and SVI were ineffective. The even greater effect of α-methyl-L-rhamnopyranoside is in accord with the structure of SVI given.

TABLE 5. *Inhibition of cross-reactions of guar and okra gums in anti-Pn VI horse sera*

Inhibitor	μMoles added	Precipitation per 1 0 ml serum by		
		Guar, from anti-VI 614	Guar, from anti-VI 681	Okra, from anti-VI 614
L-Arabinose	133			205, 80, 61*
D-Xylose	133			205, 105, 49
D-Lyxose	133			205, 100, 51
D-Mannose	111	91, 51, 44*	91, 68, 25*	
D-Glucose	111	91, 52, 43	91, 70, 23	205, 122, 41
D-Glucose	11	86, 78, 9		
L-Rhamnose	122	86, 1, 98	91, 53, 42	250, 30, 88
L-Rhamnose	12	86, 65, 24		
α-Methyl-L-rhamnopyranoside	11	85, 76, 11		
D-Galactose	111	91, 1, 99		205, 0, 100
D-Galactose	11	87, 0, 100		205, 5, 98
D-Galactose	1	87, 30, 66		245, 105, 57
D-Galactose	0.22	81, 65, 20		
α-Methyl-D-galactopyranoside	9			205, 5, 98
	0.9	85, 9, 88	91, 51, 44	
	.2	85, 57, 33		
β-Methyl-D-galactopyranoside	0.95	86, 17, 81	91, 58, 36	205, 5, 98
	.2	85, 65, 24		
Lactose	58			250, 10, 96
Melibiose	52			250, 0, 100
Ribitol	131	91, 52, 43		
Ribitol-PO_4-Na_2	39			250, 40, 84
Galactitol	1	81, 79, 2		
Sodium galactonate	1	81, 77, 5		

* The first figure gives the amount of antibody N precipitated in the absence of inhibitor, the second in the presence of inhibitor, and the third, the percentage inhibition.

These results reinforce the conclusion that the SII–SVI relationship is due to the common content of multiple residues of 1,3-linked L-rhamnose.

In the first paper of this series (Heidelberger and Rebers, 1960), the precipitation of anti-Pn VI horse sera by guar gum (literature summarized by Smith and Montgomery, 1959) and other polysaccharides containing multiple nonreducing end groups of galactose was described and a possible reason for this effect given, since SVI itself contains multiple groupings of galactose linked 1,2- rather than as end groups. It was of interest to determine the specificity of the antibody for either the alpha or beta linkage of galactopyranosides by the use of inhibitors. As seen in Table 5, galactose is the best inhibitor among the simple sugars tested, while α- and β-methyl-D-galactopyranosides are both slightly more potent and are almost equally effective. This may be due to the absence of true end groups of galactose in SVI, the determinant of anti-Pn VI specificity. Although D-galactose and its methyl pyranosides are more potent inhibitors than any of the other substances tested at very low levels, the need for caution in the interpretation of the results of inhibition of cross-reactions is seen in the strong effect of L-rhamnose. This could be due either to interaction of the inhibitor with the antibody in its specific sites, so as to hinder sterically the approach of the cross-reacting molecule, or by steric resemblance of the inhibitor to the cross-reacting galactopyranose end groups of the guar. For example, β-L-rhamnopyranose has three of its hydroxyl groups, on carbons 1, 2, and 4, in similar positions to those of α-D-galactopyranose, since both sugars are considered to assume the C-1 chair conformation (Reeves, 1951).

ACKNOWLEDGMENT

Carried out under a grant from the National Science Foundation.

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