gether with xylose, arabinose and other terminal groupings of glucuronic acid. Since there are only two parts of D- and L-galactose in 33, one might not expect strong cross-reactivity in the Type XIV antiserum. However, about 20% of the antibody is precipitated, so that the few end groups of D-galactose must be favorably placed on the molecule.

6. Guar Mucilage.—Although similar structures have been proposed for the mucilages of carob[14] and guar,[15] D-galactose constitutes about 20% of the former and 33% of the latter. Since at least 90% of the galactose in the guar mucilage, like all of that in the carob polysaccharide is in the form of non-reducing end groups, one might expect this substance to precipitate more antibody from the Type XIV antiserum than does the carob gum. Analyses at a single level of polysaccharide, near the maximum for carob,[5] showed that this is indeed true.

7. Okra Mucilage.—The mucilage of okra pods is made up of D-galactose, L-rhamnose and D-galacturonic acid.[16] Since 4-O-α(?)-D-galactopyranosyl-D-galactopyranose was isolated from the products of partial hydrolysis, only part of the galactose, if any, can be present as non-reducing end groups. Whether or not such a portion could be responsible for the small reactivity observed cannot be stated until the chemistry of this substance is studied in greater detail. However, there is no reason to suppose that α-1 → 4-linked galactose occurs in S XIV, so that if this represents the only linkage between two galactose molecules in okra mucilage, any reactivity of the substance in Type XIV antiserum would appear to be due to the presence of end groups of D-galactose.

NEW BRUNSWICK, N. J.
NEW YORK, N. Y.

[CONTRIBUTION FROM THE INSTITUTE OF MICROBIOLOGY, RUTGERS STATE UNIVERSITY]

Cross Reactions of Polyglucoses in Antipneumococcal Sera. VI.[1] Precipitation of Type VIII and Type III Antisera by β-Glucans

BY MICHAEL HEIDELBERGER AND PAUL A. REBERS

RECEIVED JULY 31, 1957

The recently elucidated structure of the immunologically specific capsular polysaccharide of Type VIII pneumococcus permitted the prediction, according to a quantitative theory of specific precipitation between antigen and antibody, that all polysaccharides containing cellobiose units would precipitate Type VIII antipneumococcal sera. This was verified for three such substances, barley and oat β-glucans, and Iles glucomannan, the only ones immediately available. It was also predicted that the two glucans, by virtue of their β-1,3-linkages, would precipitate Type III antipneumococcal serum and this also was verified.

The long-known cross-relationship between Type III and Type VIII pneumococci[2-4] was traced to the occurrence of cellobiuronic acid in the specific capsular polysaccharides[5] of both microörganisms. It was shown that S III is a polycellobiuronic acid in which each unit is joined to the next by a 1 → 3-linkage, probably β-,[6] while in S VIII only about 50% of the molecule consists of cellobiuronic acid, extra glucose being present.[4,7] In spite of the urgency of additional knowledge of the structural details of these substances upon which so promising a beginning had been made in the relation of chemical constitution to their immunological specificity, there has been no further elucidation of the stereochemistry of the linkages between the units of S III, and the fine structure of S VIII has only recently been worked out.[8] Galactose was newly discovered to be a constituent of S VIII and all residues were found to be linked 1 → 4:[·O-β-D-glucopyranosyluronic acid-(1 → 4)·O-β-D-glucopyranosyl-(1 → 4)-O-α-D-glucopyranosyl-(1 → 4)-O-α-D-galactopyranosyl-(1 → 4)-]$_n$.

It will be remembered that the absence of extensive branching in the chain of S VIII could be predicted from the quantitative studies of cross-precipitation in Type III and Type VIII antipneumococcal sera carried out many years ago.[4] The newly elucidated structure immediately made additional predictions possible. These, based also on a quantitative theory of specific precipitation in which the formation of precipitates is ascribed to multiple reactive groupings on antigen and antibody,[9] and their verification are described in the present paper.

Experimental

Materials and Methods.—S III and S VIII were supplied by E. R. Squibb and Sons, kindness of Mr. T. D. Gerlough. Type III and Type VIII antipneumococcal horse sera were furnished by the Bureau of Laboratories, New York City Department of Health, through the courtesy of Miss Annabel W. Walter. Samples of barley and oat glucans[10] were kindly supplied by Prof. Ian Preece of Edinburgh and Iles glucomannan, purified by precipitation with Fehling solution,[11] by Prof. Fred Smith of Minneapolis, Minn.

(1) Papers III, IV, V: *J. Immunol.*, **78**, 419, 427, 431 (1957). This study was carried out under a grant from the National Science Foundation to Rutgers University.

(2) J. Y. Sugg, E. L. Gaspari, W. L. Fleming and J. M. Neill, *J. Exptl. Med.*, **47**, 917 (1928); G. Cooper, M. Edwards and C. Rosenstein, *ibid.*, **49**, 461 (1929).

(3) M. Heidelberger, E. A. Kabat and D. L. Srivastava, *ibid.*, **65**, 487 (1937).

(4) M. Heidelberger, E. A. Kabat and M. Mayer, *ibid.*, **75**, 35 (1942).

(5) Herein designated S III and S VIII.

(6) M. H. Adams, R. E. Reeves and W. F. Goebel, *J. Biol. Chem.*, **140**, 653 (1941), and earlier papers.

(7) W. F. Goebel, *ibid.*, **110**, 391 (1935).

(8) J. K. N. Jones and M. B. Perry, THIS JOURNAL, **79**, 2787 (1957).

(9) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **61**, 563 (1935).

(10) I. A. Preece and K. G. Mackenzie, *J. Inst. Brewing*, **58**, 457 (1952); G. O. Aspinall and R. G. J. Telfer, *J. Chem. Soc.*, 3519 (1954); L. Acker, W. Diemair and E. Samhammer, *Z. Lebensm. Untersuch. u. Forsch.*, **102**, 225 (1955).

(11) (a) P. A. Rebers and F. Smith, THIS JOURNAL, **76**, 6097 (1954); (b) F. Smith and H. C. Srivastava, *ibid.*, **78**, 1404 (1956).

Quantitative microanalyses (Tables I and II) of the amounts of antibody nitrogen precipitated were carried out as in other papers of this series, in part with the technical aid of Mr. C. M. Soo Hoo.[12] A further improvement in technique, particularly useful during the summer months in working with cross reactions with a relatively large temperature coefficient, was to allow the tubes to drain between washings in a box surrounded with ice-water to a level higher than that of the tubes. The temperature at the level of the precipitates never exceeded 10° when this was done, and the agreement between duplicate analyses was particularly close. The cold box will therefore be used routinely in the further study of cross reactions.

TABLE I

PRECIPITATION OF TYPE VIII ANTIPNEUMOCOCCAL SERA BY HOMOLOGOUS AND CROSS REACTING POLYSACCHARIDES

0°, results calculated to 1.0 ml. of antiserum

Polysaccharide, amt. used, mg.	Antibody nitrogen precipitated from: Antiserum 1008, horse, μg.	Antiserum 644, horse, after removal of ppt. with S III, μg.	Antisera 643-5, rabbit, μg.
Pn C[a]	2		
S VIII	1288	623	2140[h]
S III	390	0	90[i]
Reduced S VIII			
0.1	704		
.2	880		
.4	1015[e]		
.8	998		
Barley glucan[b]			
0.06	122		
.09	126	55	
.2		65	231
.27	127		249
.4			208[i]
Oat glucan			
0.05	121		
0.1	125[f]		
Iles glucomannan[c]			
0.05	39		
.1			126
.15	40[g]		
.2			145
Glycogen A_{2a}[d]			
0.2	2		
1	6		
3	6		

[a] Somatic C-polysaccharide of pneumococcus. [b] Water-soluble fraction. Solution treated with solid NaCl to 0.15 *M*. [c] Dissolved with the aid of sodium xylenesulfonate (ref. 11a), dialyzed against H_2O and treated with solid NaCl to 0.15 *M*. [d] M. Heidelberger, A. Aisenberg and W. Z. Hassid, *J. Exptl. Med.*, **99**, 343 (1954); see also footnote 1. [e] Single determination. The combined supernatants from the 0.4 and 0.8 tubes failed to ppt. with S III. [f] Combined oat and barley supernatants gave no ppt. on storage in the cold, but yielded 1093 μg. of N with S VIII; calcd., 1163. With S III, 315 μg. of N pptd. [g] Combined supernatants gave 69 μg. of additional N with barley glucan. [h] Analyses by Dr. Philippe Doat. [i] The supernatant gave 96 μg. of N with Iles glucomannan, but only 134 μg. of N with barley glucan, which was evidently not used in optimal quantity. [j] The combined supernatants gave 34 μg. of N with Iles glucomannan.

Results and Discussion

As soon as it was known that the specific polysaccharide of Type VIII pneumococcus, S VIII, contained the sequence →β-D-glucopyranosyl-1 → 4-D-glucose,[8] characteristic of cellobiose,[12a] the prediction could be made that any polysaccharide containing multiple units[9] of cellobiose would give precipitates in antisera to Type VIII penumococcus. Three Type VIII horse sera and one pool of rabbit antisera were available, and all reacted quickly at 0° with both oat and barley glucans. The latter contains about one-half of its glucose in β-1,4-linkage, so that cellobiose groupings should be present. This has been shown chromatographically in the degradation of barley glucan with enzymes.[13] The structure of oat glucan is said to be similar,[10] but with a higher proportion of β-1,3-linkages. The serological findings, then, provide confirmatory evidence for the presence of units of cellobiose in the two glucans and one may confidently anticipate its isolation and chemical identification from the partially hydrolyzed substances.[13a]

TABLE II

PRECIPITATION OF C-ABSORBED TYPE III ANTIPNEUMOCOCCAL SERUM 792 BY β-POLYGLUCOSES

0°, results calculated to 1.0 ml. of antiserum, anti-III, N, 717 μg. All analyses were run with double quantities.

Polyglucose, amt. used, mg.	Antibody nitrogen pptd., μg.
Barley glucan	
0.025	17
0.075	18
Oat glucan	
0.025	20
.075	21
.15	17

In the case of Iles glucomannan, cellobiose was one of three disaccharides separated from the incompletely hydrolyzed material.[11b] The ratio of glucose to mannose in the mannan was 1:2,[11a] so that it was not surprising to find that this substance precipitated less antibody from the antisera in which quantitative analyses were run than did the barley and oat glucans.

As a check on the specificity of this reaction for β-1,4-linked D-glucose units, an oyster glycogen fraction, A_{2a} (Table I, footnote *d*), in which innumerable α-1,4-linked D-glucoses and fewer α-1,4,6-linked residues are present, was set up with a Type VIII antiserum which had given 127 μg. of N with the barley glucan. At the maximum only 6 μg. of N was precipitated (Table I). A similar experiment with jellose, a polysaccharide of tamarind seed,[14] in which two of the three glucose units are bound 1,4,6- and the third presumably 1,4-, yielded only 8 μg. of N. This permits the conclusion that in jellose the 1,4-linked glucose residues, if of the β-configuration, are either so widely or unfavorably scattered as to react only minimally with the Type VIII antibodies, or else that the linkages are of the α-1,4-configuration. Direct chemical evidence is not available. Serological differentiation between

(12) *Cf.*, also, M. Heidelberger, S. A. Barker and B. Björklund, THIS JOURNAL, **80**, 113 (1958).

(12a) W. F. Goebel (*J. Exptl. Med.*, **68**, 469 (1939)) suspected the presence of cellobiose linkages in S VIII owing to the unexpected reactivity of cellobiose-azoprotein in Type VIII antipneumococcal horse and rabbit sera.

(13) R. A. Aitken, B. P. Eddy, M. Ingram and C. Weurman, *Biochem. J.*, **64**, 63 (1956).

(13a) NOTE ADDED IN PROOF.—This has now been accomplished (personal communication from Prof. Preece).

(14) E. V. White and P. S. Rao, THIS JOURNAL, **75**, 2617 (1953).

α- and β-glycosidic linkages was first demonstrated in 1932.[15]

It will be noted from Table I, footnote *j*, that the supernatants from the precipitation of the rabbit antiserum by barley glucan gave an additional precipitate of 34 μg. of N with the Iles glucomannan, although the Iles substance precipitated only about one-half as much nitrogen from the original serum as did the glucan. It is therefore evident that the antibodies precipitated by the two substances were not entirely the same. The chemical basis for this behavior is as yet obscure. On the other hand, in antiserum 1008, from a horse, the sum of the amounts of nitrogen precipitated by successive additions of Iles and barley substances was roughly the same as with barley glucan alone. Whether or not this divergent behavior is related to the discrepancy in size between antibodies to pneumococcal polysaccharides in the horse and rabbit[16] is not clear, although quantitative studies with the smaller antibody molecules engendered in the rabbit have demonstrated degradative changes in pneumococcal polysaccharides to which the larger antibodies from the horse were insensitive.[17] It seems certain, however, that the antibodies precipitated by barley and oat glucans from antiserum 1008 were not only identical in amount, but were actually qualitatively the same. Not only have the mixed supernatants remained clear (Table I, footnote *f*) during a year's storage in the refrigerator, but they also contained an excess of the glucans, as shown by precipitation when more antiserum 1008 was added.

The quantitative data also indicate that the cross reactivity of multiples of cellobiuronic acid in the Type III–Type VIII cross reaction, and that of multiples of cellobiose are in part independent, but show some overlapping. Column 3 of Table I provides evidence that complete removal from Type VIII antipneumococcal horse serum 644 of antibodies reactive with S III[3,4] leaves much antibody precipitable by the β-glucans. Unfortunately no sample of serum 644 in its original state could be found, so that there are no data on the quantity of antibody precipitated from the whole serum. A more complete experiment with the rabbit pool (Table I, column 4 and footnote *i*) showed that more than one-half, at least, of the antibodies reactive with polycellobioses remained after removal of those precipitable by S III. An analysis in the reverse direction, of the mixed supernatants from the precipitation of horse serum 1008 by oat and barley glucans showed that 315 μg. of antibody nitrogen out of 390 was still precipitable by S III. (Table I). Deduction of 75 (390–315) from the 125 μg. of N precipitated first by the glucans leaves 50 μg. per ml. as specific for polycellobioses (Table I). It is probable, also, that the relative quantities of antibodies specific for multiples of cellobiuronic acid or cellobiose would vary considerably from one Type VIII antipneumococcal serum to another.

The specific polysaccharide of Type III pneumococcus, S III, is a salt of a polycellobiuronic acid in which each unit is linked to the next 1 → 3 from glucose to glucuronic acid. Because of the levorotation of S III, this linkage, also, is considered to have the β-configuration.[6] In this event, one would expect barley and oat glucans, which contain numerous D-glucose units with β-1,3-linkages, to show at least a small cross reaction in Type III antipneumococcal sera. That this occurs is shown by the quantitative data in Table II. If the precipitation is actually due to the β-1,3-linked glucose this would be the first confirmation of the β-configuration of the 1 → 3-linkage between D-glucose and D-glucuronic acid in S III.

Although the great importance of end groups of polysaccharides in mediating cross reactions in antisera has been demonstrated by many recent studies,[18,19] end groups have not been found in the β-glucans with which the present paper deals. One must therefore conclude either that the immunological method is more sensitive than the chemical in making such groupings evident, or else that cross reactions such as these, which involve only 10%, or less, of the total antibody, may be brought about by multiple reactive groupings at intervals along the main polysaccharide chain. The latter alternative is at least in accord with presently available information on the polysaccharides in question.

NEW BRUNSWICK, N. J.

(15) O. T. Avery, W. F. Goebel and F. H. Babers, *J. Exptl. Med.*, **55**, 769 (1932).

(16) K. Goodner, F. L. Horsfall, Jr., and J. H. Bauer, *Proc. Soc. Exptl. Biol. Med.*, **34**, 617 (1936); M. Heidelberger and K. O. Pedersen, *J. Exptl. Med.*, **65**, 393 (1937); E. A. Kabat, *J. Immunol.*, **47**, 513 (1943).

(17) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **57**, 373 (1937).

(18) Summarized by E. A. Kabat, *J. Immunol.*, **77**, 377 (1956).

(19) M. Heidelberger, THIS JOURNAL, **77**, 4308 (1955).