THE IMMUNOLOGICAL SPECIFICITY OF TYPE II PNEUMOCOCCUS AND ITS SEPARATION INTO PARTIAL SPECIFICITIES*

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It has long been known that the immunological specificities of the pneumococcal types depend mainly upon the varied chemical composition of their capsular polysaccharides (1). However, there is only scant information as to the fine structures of these substances and the relation of the component sugars of each to the resulting type specificity. The detailed structure of only the Type III specific polysaccharide has been worked out (2), and even in this instance it remains uncertain whether the linkage between the cellobiuronic acid units is in the α- or β-form at position 3 of the glucuronic acid of the adjoining unit.

In the elucidation of relationships between chemical constitution and immunological specificity, cross-reactions have proved to be of great value. The serological cross-reactivity of Types III and VIII pneumococcus (3), for example, has been traced to the occurrence of cellobiuronic acid in the specific polysaccharides of both types (4). After the introduction of quantitative, absolute microanalytical methods for the estimation of precipitins (5) this instance of cross-reactivity could be accurately and quantitatively described in weight units of precipitated antibody and theoretically accounted for (6) in terms of multiple reactive groupings on both antigen and antibody (7).

The earliest cross-reaction noted in antipneumococcal sera was, however, explicable only in general terms on the basis of knowledge available at that time. It was Dr. Oswald T. Avery's intuitive feeling that somewhere, "free in nature," were other carbohydrates with serological, and therefore chemical, properties analogous to those of the then newly discovered specific polysaccharides of pneumococcus. Accordingly, several available gums were tested

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against the only antipneumococcus sera at hand, those of Types I, II, and III. Among these combinations, gum arabic showed outstanding reactivity with the Type II horse antiserum (8), a reactivity greatly enhanced by partial hydrolysis with acid involving removal of a portion of the arabinose. Since only glucose had been identified in the Type II specific substance (S II), and the structure of gum arabic was also unknown, it could only be stated that the cross-reactivity might be due to the acidic components of both S II and gum arabic and was probably not referable to galactose, which occurred only in gum arabic.

It is the purpose of the present communication to clarify this cross-reaction in the light of the quantitative immunochemical methods already mentioned (5) and in terms of present knowledge of the fine structures of S II and gum arabic, and to discuss other instances of cross-reactivity in Type II antipneumococcus horse serum due, apparently, to the other two sugars, rhamnose and glucose, of the three which, together, constitute S II.

EXPERIMENTAL

Materials and Methods.—A commercial sample of gum arabic was used. Solutions were prepared up to a concentration of 200 mg. per ml. in 0.9 per cent saline, with neutralization as necessary, and were centrifuged to remove insoluble material. Degraded gum arabic was prepared as described in reference 8. Tamarind seed polysaccharide (jellose) (9) was kindly supplied by Dr. F. E. Brauns of the Institute of Paper Chemistry, Appleton, Wisconsin, and the gums of *Acacia pycnantha* (10) and Karaya (*Cochlospermum gossypium*) (11) by Prof. E. L. Hirst of Edinburgh.

Quantitative estimations of antibody nitrogen were carried out according to methods given in reference 5, except that amounts less than 100 μg. were usually determined by the Markham method (12, 13). All reaction mixtures were allowed to stand in a bath at 0°C. for 8 to 10 days or longer before they were centrifuged at 0°C., washed with chilled saline, and analyzed. Quantitative data are given in Table I for serum 1054, the more strongly cross-reactive of two Type II antipneumococcus horse sera of almost equal anti-II content. Data for the other serum, No. 930, are recorded in Table II. Antibody N to S II in the two sera: 1061 and 1240 μg. per ml., respectively.

Galactose and rhamnose were determined simultaneously by the 3-minute cysteine reaction (14). The carbazole method was employed for the estimation of glucuronic acid (15); galactose standards were also set up with carbazole and correction was made for the slight color given by galactose. Another variation of the reaction of cysteine with sugars, employing only the heat evolved on addition of concentrated H_2SO_4 to solutions of the samples, was used for analysis of arabinose (16). The application of this method to gum arabic will be described in a separate communication.

RESULTS AND DISCUSSION

A. Cross-Reactions in Antipneumococcus Type II Horse Serum Involving Multiple Recurrences of Glucuronic Acid.—The importance of glucuronic acid in the specificities of Types II and III was emphasized long ago by Goebel and his coworkers (17). From the quantitative data in Tables I and II it is evident that the substances which are characterized by multiple recurrences of

TABLE I

Precipitation of 1.0 Ml. Type II Antipneumococcus Horse Serum 1054 by Various Polysaccharides at 0°C.

Polysaccharides	Antibody nitrogen precipitated by								
	Lung galactan	Gum arabic		*Acacia pycnantha**	Oyster glycogen, fraction A_2	Jellose	Karaya gum*	Tb fraction*	Strep. gr. A "C"*
		Intact	Degraded						
mg.	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*
0.015						15			4‡
0.03						18§			2‖
0.05		7						3	5‡
0.075						19			
0.1			151					6	6‖
0.3			210	5	19¶			12	
0.5	95**	13					17	19‡‡	
0.75						10			
1			223		20¶				
1.5	150**	22							
2.5		32					32		
3	175**		187§§						
5		47		16			37		
10		91		26			39		
30		169		25					
100		397		21‖‖					

Analyses with 0.50 and 0.25 ml. serum, with 50 mg. gum arabic, at 2 to 3 times the relative final volume, gave 289 and 368 μg. N, respectively, calculated to 1.0 ml. serum.

* Values in these columns from serum absorbed with C substance of pneumococcus.

‡ With preparation of cell wall "C" substance kindly supplied by Dr. Maclyn McCarty. At 0.15 mg. per ml. serum this gave 2 μg. N.

§ 17 μg. N from C absorbed serum. Combined supernatants of series gave max. of 37 μg. N with karaya. Supernatants from precipitation of unabsorbed serum with both jellose and karaya gave a maximum of 137 μg. N with degraded gum arabic.

‖ Preparation C 52 C by Dr. Sverre D. Henriksen.

¶ From *J. Exp. Med.*, 1954, **99**, 343.

** From *J. Am. Chem. Soc.*, 1955, **77**, 3511.

‡‡ Supernatants gave no additional precipitate with fraction C I, obtained from fraction C by splitting off Mg palmitate with alkali. Combined supernatants from C gave, per milliliter original serum, 34 μg. N with karaya; after this, degraded gum arabic still gave 128 μg. N at the 1 mg. level.

§§ Combined supernatants of entire series chilled, recentrifuged: +0.33 mg. lung galactan per ml. original serum, gave 63 μg. N. After precipitation of 332 μg. N from the serum by intact gum arabic, SII gave 728 μg.; total, 1060, showing that the gum actually precipitated antibody N.

‖‖ The supernatants, with intact gum arabic at the same level, gave 221 μg. N.

glucuronic acid precipitate far more antibody than do those in which the cross-reactivity may be ascribed to glucose or rhamnose. This is clearly shown by the magnitude of the cross-reactions with lung galactan, already discussed in an earlier paper (18), and with gum arabic and its partial hydrolysis product. Up to 40 per cent of the total type-specific antibody may be precipitated from one of the antisera by high concentrations of gum arabic. On the other hand, the

TABLE II

Precipitation of 1.0 Ml. of Type II Antipneumococcus Horse Serum 930 by Lung Galactan and Plant Polysaccharides at 0°C.

Polysaccharide	Antibody nitrogen precipitated by				
	Lung galactan	Gum arabic		Jellose	Karaya gum
		Intact	Degraded		
mg.	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*
0.02				10	
0.03				12	4*
0.05			38	12, 11	
0.1					5*
0.15			101	11	
0.2				8	
0.3					10*
0.5			108, 121		
1	54‡				
2	70				
2.5					29
4	75				
25		114			
50		164			
100		200			

* Serum absorbed with jellose.

‡ C-absorbed serum gave 52 μg. N at this level. Supernatants from galactan-precipitated unabsorbed serum, combined, recentrifuged, gave, per milliliter original serum, 8 μg. N with degraded gum arabic, 4 μg. N with jellose, 10 μg. N with karaya. The supernatant from the last, tested further with degraded gum arabic, gave 3 μg. N.

amounts of cross-reactive antibody precipitated by polysaccharides which apparently contain the proper glucose or rhamnose linkages are an entire order of magnitude smaller.

These data might tentatively be taken to indicate that the specificity of Type II pneumococcus is principally one involving multiple recurrences of glucuronic acid and that rhamnose and glucose play a relatively minor part. Such a conclusion would certainly be strengthened by the results obtained many years ago by Marrack and Carpenter (19), who showed that partially hydrolyzed cherry gum, which contains glucuronic acid and was the most reactive of a

number of vegetable gums tested, precipitated up to 44 per cent of the antibody in the Type II antipneumococcus serum used. It must be remembered, however, that 60 and 83 per cent of the antibodies in the two antisera employed in the present study could not be precipitated by gum arabic. It is possible, moreover, that substances might be found with more favorable distributions of multiple glucose or rhamnose groupings than those used in this study and that these would precipitate more antibody. The quantitative data given on the cross-

TABLE III

Properties of Gum Arabic and Degraded Gum and their Precipitates with Type II Antipneumococcus Horse Serum 1054

Material analyzed	$S_{20} \times 10^{13}$	Arabinose	Galactose	Rhamnose	Glucuronic acid	Galactose / Rhamnose	Galactose / Glucuronic	Glucuronic / Rhamnose
		μg. per 100 μg.	*μg. per 100 μg.*	*μg. per 100 μg.*	*μg. per 100 μg.*			
Gum arabic*....	9	30,31	51, 51, 55	12, 12, 12	14, 15	4.3	3.6	1.2
Gum* recovered from specific precipitate‡...		39	49, 46	2, 3	10, 12	24, 15	5, 3.7	5, 4.1
Degraded gum, preparation A.....	1.6							
Degraded gum, preparation 66.....		6	69	9	25	7.7	2.8	2.8
Degraded gum, 66, recovered from specific precipitate§...		5	68, 68	6, 6	21, 21	11	3.2	3.5

* From the main portion of a fractionated sample.

‡ The specific precipitates contained 362 μg. total sugars and 244 μg. N.

§ The specific precipitates contained 254 μg. total sugars and 874 μg. N.

reactions therefore merely provide emphatic confirmation of the importance of multiple reactive units of glucuronic acid in Type II specificity.

Since gum arabic contains rhamnose, another constituent of S II, as well as glucuronic acid, it would be possible for this sugar to be concerned in the cross-reaction as well. In furnishing an answer to this possibility the great power of immunochemical methods is once more demonstrated. Analyses by the methods of Dische and coworkers (14–16) were made of gum arabic and its fractions and of the gum quantitatively recovered from specific precipitates with Type II antiserum after washing under the usual conditions and dissociation with trichloroacetic acid (18). As will be noted from the data in Table III, particularly the galactose: rhamnose ratios, the gum is fractionated by the antiserum. Much

of the rhamnose present fails to appear in the precipitate and two-thirds to three-quarters of it, at least, is therefore not concerned with Type II specificity. Since we have never obtained a rhamnose-free precipitate (*cf*, however, reference 20, in which are described negative results that are perhaps due to the extremely small samples analyzed), it is possible that the small residual amount of rhamnose participates in the total cross-reactivity. From the other instances in which precipitation is apparently due to rhamnose, the effect would undoubtedly be a small one. The data also show that gum arabic is a mixture, and that the single formula hitherto given for the intact gum is to that extent misleading (21).

The new data and available knowledge of the structure of gum arabic now permit an interpretation of the low titered reactivity of intact gum arabic and the high titer with which the partially hydrolyzed gum had been shown to react (8). The intact gum is characterized by a number of labile, terminal arabofuranose residues, many of which are apparently attached to glucuronic acid. The arabofuranoses constitute a hindrance to the close approach of Type II antibodies to the units of glucuronic acid and large quantities of gum must therefore be present to force maximal reactivity (Tables I and II). Even at this point, however, only 8 per cent of the gum present is precipitated. At higher dilutions of the gum, reactivity quickly fades out with reduction in the amount of precipitate and the titer is low. In the partially hydrolyzed gum, however, glucuronic acid groupings are freely exposed and many, if not all of them, are terminal. This is perhaps of significance in view of the finding of Butler and Stacey (22) that possibly one-third of the glucuronic acid in the Type II specific substance occupies the terminal position of branches of the polysaccharide. The antibody, therefore, has ready access to the groupings with which it reacts in the degraded gum, and maximal reactivity occurs at 1 mg. or less, instead of with 100 mg., as with the intact gum. The degraded gum therefore reacts to a titer at least one hundred times as great as that of the undegraded gum, as originally observed (8). The possible importance of end groupings in the reaction between antigen and antibody has recently been emphasized by Kabat (23). The precipitation of less antibody by the degraded gum than by the native material, in spite of the much higher titer of the former, need not be disturbing when a comparison is made of their sedimentation constants (Table III), for it is evident that many chain linkages have been broken in the degraded gum as well as labile arabofuranose linkages, for the molecular size is much reduced. This affords another instance of the illusory nature of titers as even a relative measure of antibody content in different systems. If the explanations given are valid, they also reduce the vague concept of "avidity" to a definite chemical picture in this one system.

The gum of *Acacia pycnantha*, which resembles gum arabic, but contains less rhamnose and roughly one-third as much glucuronic acid (10), showed much less than one-third the reactivity of gum arabic. Possibly the distribution of the

relatively few glucuronic acid groupings in the *pycnantha* gum is unfavorable for reactivity with the Type II antibody.

B. Cross-Reactions in Type II Antiserum Involving 1,4,6-Branch Points of Glucose.—The reactivity of synthetic polyglucoses (24) and glycogen (25) in Type II antipneumococcus sera has already been discussed. In view of Butler and Stacey's finding (22) that all of the glucose in S II occurs as 1,4,6-branch points the precipitation of Type II antiserum by the polyglucoses and by glycogen would seem due to their possession of such branch points suitably located for reactivity with the antibody. Just as the reactivity of glycogen in the antiserum had been predicted from Butler and Stacey's finding and from the quantitative theory of the precipitin reaction (7), it was possible to predict that tamarind seed polysaccharide, or jellose, in which two-thirds of the glucose is in the form of 1,4,6-branch points, would also react. The experiments recorded in Tables I and II show the confirmation of this prediction. It may be seen from Table I that the same quantity of antibody is precipitated by jellose as by oyster glycogen. Possibly 1,6-linkages, in these instances at the 1,4,6-branch points, suffice for the limited extent of cross-reactivity.

C. Cross-Reactions in Type II Antiserum Apparently Involving Rhamnose.—The only rhamnose derivative isolated by Butler and Stacey (22) from methylated S II was 2,4-di-O-methyl-L-rhamnopyranose, indicating that all of the rhamnose is linked in positions 1 and 3. This might be considered another reason for excluding the small portion of the rhamnose of gum arabic carried down in the Type II precipitate from participation in the reactivity of the gum, for the rhamnose is usually shown as end groups (21). However, in the high rotating polysaccharide of a human strain of tubercle bacillus, fraction C (26), all of the rhamnose, the only sugar present which occurs in S II, is also assigned to end positions (27), yet the C fraction reacts with Type II antiserum to about the same extent as glycogen.

It will be noted, also, that reaction occurs with the C substance of hemolytic streptococci, Group A. This substance is said (28) to consist of five parts of rhamnose to two of glucosamine. Nothing is known of its fine structure.

Another substance which presumably reacts with Type II antiserum because of its content of L-rhamnose is karaya gum (from *Cochlospermum gossypium*). The sugars identified (11) in this complex product are, in equimolecular proportions, L-rhamnose, D-galactose, and D-galacturonic acid, with traces of a ketohexose. The amounts of antibody precipitated by this gum are somewhat greater (Tables I and II) than the quantities precipitated by the other two polysaccharides in which the reactivity is also ascribed to rhamnose.

It is evident that the antibodies which react with the partial specificities described above do not exceed 50 per cent for serum 1054 and 20 per cent for serum 930. While these figures lack absolute significance, it is clear that a large proportion of the antibodies in both antisera require, for specific precipitation,

more than merely multiple recurrences of suitably linked single sugar components of S II. Unfortunately, with the possible exception of gum arabic, there were no carbohydrates available with a content of more than one of the components of S II in proper linkage.[1] On the basis of the present material, then, the sum of the partial specificities does not equal the whole, and much of the antibody, in different amount in each antiserum, is more rigorously type-specific than the portions dealt with in this communication. The present paper, then, also contributes to the store of quantitative information on the heterogeneity of antibodies.

SUMMARY

The specificity of Type II pneumococcus determined by its capsular polysaccharide (S II) may be separated into three partial specificities, each characteristic of one of the three component sugars of S II, namely, glucuronic acid, glucose, and rhamnose.

By far the largest portion of antibodies in Type II antipneumococcus horse sera which cross-react with carbohydrates containing one or more of these sugars are reactive with those characterized by multiple groupings of glucuronic acid. This confirms, extends, and explains earlier observations.

In confirmation of predictions based upon existing information tamarind seed polysaccharide (jellose), in which much of the glucose exists as 1,4,6-branch points, was found to react in Type II antisera.

Several instances are given of cross-reactions in these antisera apparently due to the L-rhamnose component of the reacting polysaccharides.

The antisera contain far more antibody capable of precipitating with substances with multiple units of glucuronic acid than with those so far tested containing multiple 1,4,6-branch points of glucose or multiple groupings of rhamnose.

The long known increase of titer of gum arabic on partial hydrolysis is now fully explained and discussed, and a chemical picture is given of the change in "avidity."

The sum of the partial specificities measured does not equal the whole.

Quantitative data illustrating these points are given.

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[1] S XVIII contains both glucose and rhamnose (29), but obviously unsuitably linked because there is no cross-reactivity between Types II and XVIII in either direction.

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