A QUANTITATIVE STUDY OF THE CROSS REACTION OF TYPES III AND VIII PNEUMOCOCCI IN HORSE AND RABBIT ANTISERA*

By MICHAEL HEIDELBERGER, Ph.D., ELVIN A. KABAT, and D. L. SHRIVASTAVA,** D.Sc.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

(Received for publication, January 20, 1937)

Atypical Type III strains of pneumococci were first isolated by Sugg, Gaspari, Fleming, and Neill (1). These strains gave cross reactions with Type III antisera, but failed to remove all of the antibody. Cooper, Edwards, and Rosenstein (2) classified these atypical strains as Type VIII and found that incomplete crossing occurred in antisera to both types. Paralleling the immunological relationships is a close similarity of the type specific polysaccharides. Thus the Type III substance is a polyglucuronoglucose (3), while Goebel has found the Type VIII substance to contain the same aldobionic acid (glucuronoglucose and glucose in addition (4).

The development of quantitative chemical methods for the micro estimation of precipitins (5, 6) and agglutinins (7) offered an accurate means of studying the extent and nature of this cross reaction. In the course of this study the specific polysaccharide of Type VIII pneumococcus was prepared by the method recently published from this laboratory (8).

EXPERIMENTAL

Materials.—Type III and Type VIII horse and rabbit antipneumococcus sera were used in this investigation.[1] Sera H 636, H 607, R 3.50_2, and R 3.49_2 were

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York.

** Rockefeller Foundation Fellow, 1934–1936.

[1] The horse sera were kindly supplied by Dr. William H. Park and Miss Georgia Cooper of the New York City Department of Health Laboratories, and the Type VIII rabbit sera by Drs. Goodner and Horsfall of the Hospital of The Rockefeller Institute for Medical Research.

absorbed with C substance and R protein derived from a Type I strain of pneumococcus. The bacterial suspensions used for the agglutinin determinations were prepared as described in Reference 7. The Type III and Type VIII pneumococcus polysaccharides (hereafter referred to as S III and S VIII) were isolated without the use of heat, strong acid, or alkali (8).

In the preparation of S VIII,[2] it was found that after the initial alcohol precipitation of the material from the vacuum concentrate of 10.5 liters of a 16 hour broth culture, it was necessary to use only 0.5 volume of alcohol as precipitant in the presence of sufficient sodium acetate and acetic acid. The yield was 0.61 gm. of the dried sodium salt. Its properties are given below and are in essential agreement with the portions of the data given by Brown (9) and by Goebel (4). From the high viscosity, it is to be presumed that the new preparation is in a less degraded form than the earlier products in which heat was used (*cf.* 8). The S VIII contained a small, but serologically demonstrable, amount of C substance.

Properties of the Type VIII Pneumococcus Polysaccharide

Ash as Na	N*	$[\alpha]_D^{26}$*	Neutral equivalent†	Acetyl*	Uronic anhydride*	Reducing sugar on hydrolysis*	η_{rel} 0.10% solution in 0.9% NaCl	η_{rel} 0.20% solution in H_2O
per cent	*per cent*	*degrees*		*per cent*	*per cent*	*per cent*		
3.1	0.2	+123	720‡	0.5	27‡	87	2.50	16.6

* Calculated to the ash-free basis.

† Calculated from Na content.

‡ Calculated for $[(C_6H_{10}O_5)_3(C_6H_8O_6)]_x$: Neutral equivalent, 662; Uronic anhydride, 26.6%.

The precipitin determinations were carried out by addition of known amounts of specific polysaccharide to accurately measured quantities of serum and determination of the specifically precipitable nitrogen after 48 hours at 0° in the washed precipitates by the micro Kjeldahl method (5, 6). Agglutinin determinations were made similarly (7), using measured volumes of washed, heat-killed pneumococcus suspension and estimating the increase in nitrogen content. All determinations were run, in duplicate, at 0° for 48 hours to remove antibody as completely as possible. The results are summarized in Table I. Under the columns headed Precipitin N and Agglutinin N are listed the values for the maximum amount of antibody found, using the specific polysaccharide and the bacterial suspension, respectively. To estimate the group specific antibody present, agglutinin determinations were carried out with a pneumococcus I R (Dawson S) suspension. In the last column are tabulated the sums of the agglutinin found, using the heterologous strain first, followed by determination of the remaining

[2] The Type VIII pneumococcus strain was kindly supplied by Dr. O. T. Avery of the Hospital of The Rockefeller Institute.

antibody on an aliquot portion of the supernatant with a suspension of the homologous type. Since this procedure involved up to four successive sets of analyses on the same solution, agreement with the directly determined homologous agglutinin N was not always perfect.

In Table II are given data on the addition of increasing amounts of S VIII and S III to Type VIII horse antiserum, H 644. Before setting up these experi-

TABLE I

Quantitative Agglutinin and Precipitin Determinations on 1 Ml. of Horse (H) and Rabbit (R) Antisera to Pneumococcus III and VIII

Serum No.	Serum type	Antipneumococcus III content		Antipneumococcus VIII content		Anti-I R	Heterologous agglutinin N + homologous agglutinin N
		Precipitin N	Agglutinin N	Precipitin N	Agglutinin N	Agglutinin N	
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
H607, 1:1	III	0.68	0.75	0.11	0.22	0.11	0.79
H636	VIII	0.33	0.40	0.99	1.20	0.11	1.15
H644, 2:5*	VIII	0.55	0.65	1.46	1.42	0.35	1.30
$R3.50_2$	III	2.38	2.43	0.00	0.09	0.03	
$R3.49_2$	III	0.76	0.86	0.04	0.23	0.15†	
R7.18	VIII	0.06	0.25	>1.36	1.59‡	0.10	1.56
R7.19	VIII	0.00	0.20	0.84	1.21‡	0.11	1.10
		Antipneumococcus I content					
R3.70, 1:2.5	I	0.43§	0.45				

* This serum still contained anti-C. Since the S VIII contained C substance, the relatively high precipitin N value is accounted for.

† The supernatant, now free from anti-C, gave a definite precipitate with S VIII.

‡ The supernatants from the Type III precipitin determinations were used. 0.06 was therefore added to the agglutinin found in the R 7.18 serum. Owing to the small quantities of the Type VIII rabbit sera available, analytical samples of 0.5 ml. were used. The usual error was therefore doubled.

§ The supernatant, set up with pneumococcus I S suspension, yielded no more agglutinin N.

ments, 16 ml. of the serum were diluted to 40 ml. and absorbed with C substance, so that only precipitin for the type specific carbohydrates would be measured. In order to ascertain whether or not precipitation of the Type VIII serum with S III resulted in a change in the quantitative relationships of the residual antibody, the supernatants from the S III precipitations were combined, treated with an excess of S III, and again centrifuged. Portions of the supernatant were then set up with S VIII as in Table II. Calculations were made from the data as in

TABLE II

Antibody N Precipitated from Type VIII Antiserum H 644 by Varying Amounts of S VIII and S III

Amount specific polysaccharide used	Antibody N precipitated by S VIII from 1.0 ml. serum dilution	Ratio antibody N precipitated to S VIII used	Tests on supernatants	Antibody N precipitated by S III from 2.0 ml. same serum dilution	Ratio antibody N precipitated to S III used	Tests on supernatants	Antibody N precipitated by S VIII from 1.5 ml. serum dilution freed from cross reacting antibody	Ratio antibody N precipitated to S VIII used	Tests on supernatants
mg.	*mg.*			*mg.*			*mg.*		
0.020							0.362	18.1	Excess A
0.030				0.400	13.6	Excess A†	0.508	16.7	" "
0.050	0.820	16.4	Excess A*				0.630	12.6	" "
0.059				0.502	8.5	" "			
0.075	1.036	13.8	" "				0.676	9.0	No A or S
0.089				0.578	6.5	" "			
0.100	1.128	11.3	Trace A (?)				0.678	6.8	" " " "
0.118				0.654	(5.7)	Trace S			
0.150	1.116	7.4	No A or S				0.670		Excess S
0.177				0.736		Excess S			
0.200	1.110		Excess S				0.670		" "
0.236				0.752		" S			
Equation:	mg. N pptd. = 21.4 S − 101 S²						mg. N pptd. = 21.4 S − 167 S²		
	S max. = 0.106‡						S max. = 0.064‡		
	A max. = 1.136‡						A max. = 0.685‡		

The S III-anti-S VIII reaction is inhibited by high concentrations of S III.

* A = antibody.

† In addition there was no precipitate with H 644 serum or C-absorbed pneumococcus III rabbit antiserum, showing absence of S III in supernatant.

‡ Values calculated from the equation.

(10) and it was found that the general equation there derived held for the reaction between S VIII and its homologous antibody. The data are also plotted in Fig. 1 as recalculated to 1.00 mg. of antibody N so that the different experiments may be compared. The equations used are:

$$\text{mg. antibody N pptd.} = 2RS - \frac{R^2}{A} S^2, \text{ and}$$

the linear relation,

$$\frac{\text{mg. antibody N pptd.}}{S} = 2R - \frac{R^2}{A} S,$$

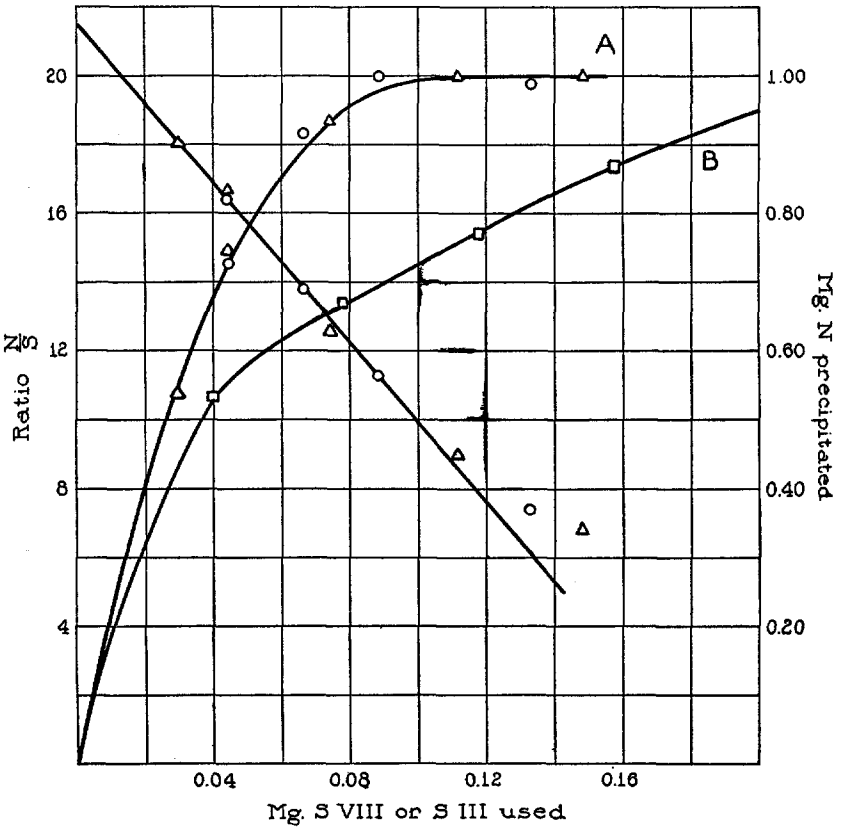
in which R is the ratio of antibody N to S at a reference point in the equivalence zone and A is the amount of antibody precipitated at that point. It will be noted that the cross reaction fails to follow these equations.

DISCUSSION

The quantitative study of the homologous and cross reactions of Types III and VIII pneumococcus and their respective antisera derived from the horse and from the rabbit has revealed several points of interest. As will be seen from Table I, homologous precipitin is, in general, approximately equal to homologous anticarbohydrate agglutinin both in the horse and rabbit antisera, if the antibody reactive with pneumococcus I R be deducted. This not only furnishes additional instances (*cf.* 11) of the quantitative correspondence of anticarbohydrate precipitin and agglutinin but indicates also that the specific polysaccharides used as reagents for precipitin were isolated (8) in a form in which their reactivity differs little from their behavior toward antisera when present in or on the bacterial cell. This is the more significant since S I prepared by Chow's recent modification of the above method employing trichloroacetic acid (12) is stated by this worker to precipitate only 35 per cent of the antibody in a Type I antipneumococcus rabbit serum. S I prepared according to Reference 8 precipitates practically all of the anticarbohydrate in rabbit serum as well as in horse serum (11), as shown in the last line of Table I.

As for the cross reactions, the results shown in Table I are somewhat complicated by the presence of group specific antibody even in the sera which had been absorbed with C substance and R protein.

The amount of this antibody is shown in Table I under Anti-R agglutinin. In spite of this it is evident that a larger proportion of



TEXT-FIG. 1. Reaction of S VIII and S III in antipneumococcus VIII horse serum, absorbed with C substance.

○, S VIII-anti-S VIII reaction.

□, S III-anti-S VIII reaction.

△, S VIII-anti-S VIII reaction after removal of antibody precipitated by excess S III.

All reactions calculated to 1.00 mg. of total antibody N.

the anticarbohydrate in the horse sera was cross reactive in both directions than in the rabbit sera, and that the two anti-VIII horse sera tested contained a higher proportion of cross reacting anticarbo-

hydrate than did the anti-III serum. Of the rabbit sera, one Type III and one Type VIII serum failed to show crossing with the heterologous specific polysaccharide, a failure which did not depend upon the total antibody present, since the non-reactive III serum (R 3.50_2) contained three times as much antibody as did the cross reacting III serum (R 3.49_2). Neill and his associates (1) also noted variations in the cross reactivity of both horse and rabbit antisera which did not depend on the homologous antibody titer.

A possible practical application of the data summarized in Table I would be the absorption of diagnostic Type III and Type VIII antisera with Type VIII and Type III S (Dawson M) pneumococci, respectively, in order to eliminate crossing due to S III, S VIII, and group specific components.

As shown in Table II and Fig. 1 (curve A and the line) the precipitin reaction between the specific polysaccharide of Type VIII pneumococcus (S VIII) and its homologous antibody may be quantitatively expressed, in the region of excess antibody, by an equation of the same form as that derived for the Type III reaction (10), and would therefore appear to be governed by the same mechanism. The cross reaction between the Type III pneumococcus polysaccharide (S III) and Type VIII antibody, however, appears to be of a different nature, and the curve (B) obtained by plotting the amount of antibody nitrogen precipitated against S III added is of a different form. It will be noted (Table II) that a large excess of S III is required in order to precipitate all of the cross reacting antibody. While this recalls in some measure the behavior of crystalline egg albumin in the cross reaction with the antibodies to R-salt-azo-biphenyl-azo egg albumin (13), the S III-anti-S VIII compounds formed gave no evidence of the dissociation which was so marked in the egg albumin-anti-dye cross reaction. This was shown by the failure of the supernatants from the S III-anti-S VIII precipitates in the region of excess antibody to react with either Type III or VIII antiserum which had been absorbed with C substance. An additional difference is the inhibition of precipitation caused by a large excess of S III. The two cross reactions of which a quantitative study has been made are therefore of quite different character, and this is also brought out by the relatively small proportion of cross reacting antibody in the present

instance. It is also striking that the 70 per cent of residual, strictly type specific anti-S VIII, after removal of the antibody precipitated by S III, followed exactly the same equation, when calculated to the same antibody content (see Fig. 1) as did the whole antibody. This would indicate that no fractionation had occurred with respect to reactivity with S VIII, and that the antibody removed by S III had the same quantitative relationship to S VIII as did the portion which did not react with S III.

It is believed that the quantitative data accumulated in the present study also throw light on certain of the chemical and immunobiological factors concerned in pneumococcus type specificity and the cross reaction between types. It will be noted from Table I that the rabbit antisera studied contain little or no cross reacting antibody, although their content of antibody reactive with the homologous polysaccharide is as large as that of the horse sera. Indeed, a rabbit Type III antiserum, 3.50_2, with the unusually high titer of 2.4 mg. of homologous anticarbohydrate nitrogen per ml., failed entirely to precipitate S VIII. Closely related as are S III and S VIII chemically in their content of the same structural unit (4), the pneumococcus antigens of which these polysaccharides form the determinants of specificity are capable of eliciting in the rabbit abundant production of strictly type specific antibody. Although this antibody reacts quantitatively as completely with the homologous specific polysaccharide as it does with the entire antigen (as, for example, heat-killed pneumococci of the same type), it is inert, or nearly so, toward the closely related polysaccharide or antigen of the other type. This, it is believed, indicates that the true type specific antibody is directed toward multiples of the characteristic chemical structural unit of the homologous specific polysaccharide. The implications of this interpretation will be discussed in greater detail when an experimental test of its validity, now under way, has been completed. It would appear, however, that the common aldobionic acid component of the two polysaccharides, a glucuronoglucose, is concerned in the true type specific response only as part of a larger, type characteristic structural unit.

As for the cross reaction, there seems no reason to alter the view of Avery, Heidelberger, and Goebel (14) that cross reacting polysaccharides "contain in a portion of the complex molecule the same or

a closely similar configuration of atoms." It would appear premature to attempt to localize even the cross reactivity in a more definite portion of the common configuration. Goebel, for example, has emphasized the importance of the uronic acid portion of the aldobionic acid (4, 15), but Avery and Goebel (16) have shown that the rotation of a single carbon atom through an angle of 180°, as in glucose and galactose, produced as profound a change in specificity as did the substitution of a terminal —COOH group, as in glucuronic acid, for —CH_2OH, as in glucose (15). Moreover, precipitation of an antipneumococcus Type III horse serum with an artificial glucuronic acid antigen (15) must either have removed antibody unrelated to the cross reaction with S VIII, or else removed a quantitatively insignificant amount of antibody, since it failed to reduce the qualitative cross reaction titer to S VIII.

SUMMARY

1. A preparation of the specific polysaccharide of Type VIII pneumococcus is described in which the use of heat, strong acid, and alkali were avoided.

2. Quantitative estimations are given of the homologous and cross reacting precipitin and agglutinin in Types III and VIII antisera produced in the rabbit and in the horse. Quantitative data are also given on the mechanism of the Type VIII precipitin reaction and the cross reaction between the Type III polysaccharide and Type VIII antipneumococcus horse serum.

3. The significance of the data is discussed.

BIBLIOGRAPHY

1. Sugg, J. Y., Gaspari, E. L., Fleming, W. L., and Neill, J. M., *J. Exp. Med.*, 1928, **47**, 917.
2. Cooper, G., Edwards, M., and Rosenstein, C., *J. Exp. Med.*, 1929, **49**, 461.
3. Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, 1926, **70**, 613; 1927, **74**, 613.
4. Goebel, W. F., *J. Biol. Chem.*, 1935, **110**, 391.
5. Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M., *J. Exp. Med.*, 1933, **58**, 137.
6. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 559.
7. Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1934, **60**, 643.

8. Heidelberger, M., Kendall, F. E., and Scherp, H. W., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33,** 188, 445; *J. Exp. Med.*, 1936, **64,** 559.
9. Brown, R., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32,** 859.
10. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61,** 563.
11. Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1936, **63,** 737.
12. Chow, B. F., *J. Exp. Med.*, 1936, **64,** 843.
13. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1934, **59,** 519.
14. Avery, O. T., Heidelberger, M., and Goebel, W. F., *J. Exp. Med.*, 1925, **42,** 709.
15. Goebel, W. F., *J. Exp. Med.*, 1936, **64,** 843.
16. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **50,** 533.