A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

IV. The Reaction of Pneumococcus Specific Polysaccharides with Homologous Rabbit Antisera*

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It has been shown that the precipitin reaction between the specific polysaccharide of Type III pneumococcus and homologous antibody produced in the horse takes place according to the equation

$$\text{mg. antibody N precipitated} = 2RS - \frac{R^2}{A} S^2 \quad \ldots\ldots\ldots\ldots\ldots [1]$$

in which S is the amount of specific polysaccharide used and R is the ratio of the components in the precipitate at a reference point in the equivalence zone (1). It was found that this equation could be derived from the mass law if the reaction were considered the resultant of competing bimolecular reactions, and that in the derivation the volume factors cancelled, in agreement with the experimental observation that the composition and amount of the precipitate depended on the relative proportions in which the components were mixed, rather than on their concentrations. The formation of the precipitate was considered due to the building up of large aggregates by the union, according to the equation, of multivalent S with multivalent antibody.

It was also shown that this quantitative theory of the precipitin reaction was applicable to antigen-antibody systems (2, 3) as well as to the hapten-antibody reaction. Since differences had been observed between the behavior of antibodies produced in the horse and those

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derived from the rabbit toward specific polysaccharides and their degradation products (4), a study was initiated several years ago of the reaction between S III[1] and homologous antibody produced in the rabbit. However, only recent data are included in the present report, since it was found that pneumococcus specific polysaccharides were not thermostable, as had been supposed, and those used in the earlier work failed to yield maximal precipitates with rabbit antibody (5).

EXPERIMENTAL

Antisera.—Rabbits were injected intravenously with Type III pneumococci in the mucoid phase, strain A 66, killed by the addition of 2 per cent by volume of 40 per cent formalin solution. The suspension injected contained 0.05 mg. of bacterial nitrogen per ml. The immunizing dose was increased from 1 ml. to 3 ml. during a course of 15 injections, of which 3 to 4 were given per week. The initial bleedings, 3.50_1 and 3.51_1, were made 4 days after the last injection. After a rest period of 1 week a second course of 12 injections was given, bleedings 3.50_2 and 3.51_2 being taken 7 days after the last injection. Third and fourth courses of injections were given similarly. In these two rabbits the sera from the first course contained the largest amounts of antibody.

Rabbit 3.70 was given one course of acid-killed pneumococcus Type I suspension, strain I 230, but did not yield a potent antiserum until after nearly 40 additional injections of formalinized suspension.

Before use the sera were precipitated with C substance (6) in excess, in order to remove antibody which would react with traces of this substance remaining in the polysaccharide used.

Specific Polysaccharides of Types I and III Pneumococcus.—These substances were prepared without the use of heat, alkali, or strong acid, according to (5), since earlier methods of preparation yielded products with impaired ability to precipitate rabbit antisera.

Determination of the Antibody Nitrogen Precipitated by Various Amounts of S I and S III.[1]—An accurately measured quantity of antiserum containing between 0.65 and 1.25 mg. of antibody nitrogen was added to various amounts of polysaccharide in saline at 0°, at a final volume of 4 ml. After 48 hours in the ice box the precipitates were centrifuged, washed with saline, and analyzed for nitrogen by the micro Kjeldahl method as described in earlier papers (7, 8). All supernatants were tested for both components by the addition of antiserum and polysaccharide to separate portions. Polysaccharide was estimated quantitatively in many of the supernatants according to (9).

[1] S I and S III are used throughout to designate the specific polysaccharides of Types I and III pneumococcus.

In Table I are given data on the solubility of the S III-anti-S III precipitate and the effect of temperature on the amount of antibody nitrogen precipitated. In Table II are given the amount of antiserum used, the quantity of antibody N precipitated by the given amounts of S III, the amount of nitrogen calculated from the equation for the reaction, the ratio between antibody N and S III in the precipitate, the tests on the supernatants, and notes on the appearance of the precipitates. Corresponding data for S I and serum 3.70 are given in Table V.

TABLE I

Effect of Temperature and Dilution on Antibody Precipitated from Type III Antipneumococcus Rabbit Sera

Antiserum	Volume used	Amount of S III	Final volume	Antibody N precipitated		Tests on supernatants
				at 37°	at 0°	
	ml.	*mg.*	*ml.*	*mg.*	*mg.*	
$3.49_1(1:1)$	1.0	0.10	2.0	0.50	0.61	Excess S
$3.51_2(1:1)$	1.0	0.060	2.0	0.50	0.55	Excess A
	1.0	0.079	2.0	0.55	0.61	Excess S
						Difference per ml.
						mg.
$3.49_1(1:1)$	1.0	0.10	2.0		0.550*	
	1.0	0.10	10.0		0.494*	0.007
$3.50_2(2:1)$	1.0	0.15	2.0		0.796*	
	1.0	0.15	12.0		0.736*	0.006
$3.51_2(1:1)$	1.0	0.06	2.0		0.496*	
	1.0	0.06	9.0		0.474*	0.003†

* Run in triplicate. The supernatants were also centrifuged and any additional traces of precipitate were washed with the washings from the main portions.

† In this series the larger tubes were washed with 3.5 ml. more saline than were the smaller tubes. Correcting for this, the difference per ml. is reduced to 0.002 mg.

DISCUSSION

Although antisera produced in the rabbit were included in earlier quantitative studies (2, 3), the present series of experiments permits for the first time a comparison of the behavior in the precipitin reaction of antisera produced with the same antigen in the horse and in the rabbit. The data summarized in the tables indicate that the

TABLE II

Addition of S III to Serum 3.51

Amount S III added	Antibody N precipitated	Antibody N calculated from equation	Experimental ratio N precipitated to S III precipitated	Character of precipitate	Tests on supernatants
mg.	*mg.*	*mg.*			
Course 1. Serum diluted with 5 volumes saline. 1.0 ml. samples used					
0.02	0.217	0.23	10.9		Excess A
0.04	0.424	0.41	10.6		Excess A
0.06	0.544	0.54	9.1		Excess A
0.08	0.617	0.62	7.7		No A or S
0.10	0.664*	0.66	6.6		No A or S
0.12	0.680		5.8†		Trace S
0.15	0.687				Excess S
0.177	0.682		4.1†		Excess S
0.20	0.696				Excess S
0.30	0.660				Excess S
0.50	0.619				Excess S
1.00	0.255				Excess S
1.50	0.064				Excess S
Equation: Mg. antibody N pptd. = 12.7 S − 61.1 S^2. S max. = 0.104 mg. A max. = 0.661 mg. N					
Course 2. Serum diluted with equal volume saline. 1.0 ml. samples used					
0.02	0.251	0.27	12.5	Op., Fl.	Excess A
0.04	0.436	0.44	10.9	Op., Fl.	Excess A
0.06	0.550	0.56	9.2	Op., C.	Trace A
0.08	0.588	0.60	7.4	Tl., C.	Trace S
0.10	0.614			Jelly	Excess S
0.12	0.644		5.6†	Jelly	Excess S
0.20	0.638			Jelly	Excess S
0.50	0.504*			Jelly	Excess S
1.00	0.124			Jelly	Excess S
1.50	0.004				
Equation: Mg. antibody N pptd. = 14.5 S − 87.1 S^2. S max. = 0.084 mg. A max. = 0.600 mg. N					

Op. = opaque; Fl. = flocculent; C. = compact; Tl. = translucent.

* One determination only.

† Calculated after analyzing supernatant for S according to (9) and deducting amount found from that used.

TABLE II—*Concluded*

Amount S III added	Antibody N precipitated	Antibody N calculated from equation	Experimental ratio N precipitated to S III precipitated	Character of precipitate	Tests on supernatants
mg.	*mg.*	*mg.*			
Course 3. Serum diluted with 0.5 volume saline. 1.0 ml. samples used					
0.02	0.292	0.30	14.6	Op., Fl.	Excess A
0.04	0.554	0.54	13.9	Op., Fl.	Excess A
0.06	0.766	0.74	12.8	Op., Fl.	Excess A
0.08	0.866*	0.88	10.8	Op., Fl.	Excess A
0.10	0.926	0.97	9.3	Op., C.	Excess A
0.15	0.982*	0.98	6.5	Op., C.	No A or S
0.20	0.996				Excess S
0.50	0.940			Jelly	Excess S
1.00	0.602			Jelly	Excess S
1.50	0.362			Jelly	Excess S
2.00	0.336			Jelly	Excess S
Equation: Mg. antibody N pptd. = 16.1 S − 63.9 S^2. S max. = 0.126 mg. A max. = 1.015 mg. N					
Course 4. 1.5 ml. samples undiluted serum used					
0.02	0.298	0.30	14.9		Excess A
0.04	0.518	0.51	13.0		Excess A
0.06	0.652*	0.66	10.9		Excess A
0.08	0.724	0.72	9.1		No A or S
0.50	0.704				Excess S
1.00	0.386				Excess S
1.50	0.070				Excess S
Equation: Mg. antibody N pptd. = 16.7 S − 96.5 S^2. S max. = 0.087 mg. A max. = 0.722 mg. N					

Calculated to 1.000 mg. of antibody N, these four equations become:

For 3.51_1: mg. antibody N pptd. = 12.7 S − 40.3 S^2

" 3.51_2: mg. antibody N pptd. = 14.5 S − 52.6 S^2

" 3.51_3: mg. antibody N pptd. = 16.1 S − 64.8 S^2

" 3.51_4: mg. antibody N pptd. = 16.7 S − 69.7 S^2

For serum 3.50, calculated to 1.000 mg. of antibody N, the following were found:

" 3.50_1: mg. antibody N pptd. = 14.9 S − 46.2 S^2

" 3.50_2: mg. antibody N pptd. = 15.6 S − 60.8 S^2

" 3.50_3: mg. antibody N pptd. = 16.8 S − 70.6 S^2

reaction between homologous polysaccharide and antibody in rabbit Type III antipneumococcus sera follows the same general course as

in sera produced in the horse (1) and may also be quantitatively described by expressions of the form of Equation 1 derived from the mass law.

From Table I it will be noted that the effect of temperature on the S III-antibody reaction is much the same in the sera of the two animals in question (*cf.* (1) for the reaction with horse antibody), less antibody being precipitated at 37° than at 0°. However, the solubility of the specific precipitate at 0° is considerably greater in the rabbit antisera than in horse antisera and is about the same as that of the egg albumin-antibody precipitate (3). Subject to this correction, the amount of antibody precipitated does not depend on the final concentrations of the reactants, but on the relative proportions in which they are mixed, just as in the other systems studied.

In Table II are given data on the addition of increasing amounts of S III to sera obtained from the same rabbit after four successive courses of intravenous injections of formalinized Type III pneumococci. While inconstant results were obtained with earlier preparations of S III and the equivalence zone was characterized by the simultaneous presence of both polysaccharide and antibody, it will be noted that with S III prepared without the use of heat, strong acid, or alkali, as in (5), neither antibody nor polysaccharide could be detected in supernatants in the equivalence zone. When the ratios of antibody N to S III precipitated in the region of excess antibody were plotted against S III and the best line, calculated by the method of least squares, was drawn through the points, the equation of the line

$$\frac{N}{S}_{\text{precipitated}} = 2R - \frac{R^2}{A} S \quad [2]$$

described the behavior of the serum in the region of excess antibody and was converted into Equation 1 by multiplying through by S. Equations of this form, which give the amount of antibody N precipitated by any quantity of S up to the maximum, are given below the data for the serum from each course. Comparison of the found and calculated antibody values (columns 2 and 3, Table II) shows close agreement.

At the foot of Table II the equations for the four courses are compared at the same antibody content. It will be noted that R increases during immunization, as had previously been noted in the case of a rabbit injected with egg albumin (3). As also noted at the foot of Table II, R increased similarly during the immunization of another rabbit, 3.50. The data in Table III show that the amplitude of the equivalence zone does not appear to increase as definitely on progressive immunization as in the egg albumin system. It

TABLE III

Equivalence Zone Ratios of Antibody N to S in Precipitate

Serum	Observed R at A excess end of zone	R from equation	Observed R at S excess end of zone
Rabbit 3.50_1 Type III	7.9	7.5	6.3
" 3.50_2 " "	8.9	7.8	6.4
" 3.50_3 " "	10.5	8.4	6.8
" 3.51_1 " "	(8.5)	6.4	(6.1)
" 3.51_2 " "	9.2	7.3	7.4
" 3.51_3 " "	(8)	8.1	(6)
" 3.51_4 " "	(10)	8.4	
" 3.48_1 " "	(5.5?)	6.5	(5)
Mean		7.6	
Corresponding R from antipneumococcus III horse serum 792 at 0° with S III prepared according to Ref. 5	11.3	10.6	6.1
Rabbit 3.70 Type I	(3.0)	2.7	(2.7)
Corresponding R from horse antipneumococcus I solution at 0° with S I prepared according to Ref. 5	(6.8)	7.2	(4.0)

Values in parentheses deduced from nearest actual determinations.

appears probable, nevertheless, that the observed increases in R in the later stages of immunization are due to the formation of antibody reactive with additional chemical groupings on the S III molecule which did not react with the antibody produced in the earlier stages of immunization.

From Table IV it will be seen that the relation

$$\text{mg. S III precipitated} = 2R'A - \frac{(R')^2}{S}A^2 \quad [3]$$

in which S is the total amount of S III added and R′ is the ratio of S III to antibody N in the precipitate at the S III excess end of the equivalence zone, holds moderately well for the region of polysaccharide excess up to the beginning of the inhibition zone. As will also be noted from Table II, the extent of the region of maximum precipitation is quite limited, and the inhibition zone begins with smaller amounts of specific polysaccharide than necessary for Type III antipneumococcus horse sera. However, in the region of S III excess there appears to be little difference in the composition of the specific precipitate in the rabbit and horse sera, as the ratios of the components are much the same in both (Tables II and III, also Table III, reference 1), except possibly toward the beginning of the inhibition zone, at which N:S ratios as low as 3.8 and 3.5 were found for

TABLE IV

Calculated and Found Values of S III Precipitated in Region of Excess Polysaccharide

Serum	S III added	S III found in precipitate	S III calculated in precipitate	Serum	S III added	S III found in precipitate	S III calculated in precipitate
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
3.48_1	0.167	0.163	0.167	3.50_3	0.118	0.117	0.118
(R′ = 0.2)	0.200	0.192	0.193	(R′ = 0.147)	0.177	0.171	0.156
(A = 0.81)	0.225	0.211	0.207	(A = 0.79)	0.236	0.199	0.175
	0.250	0.232	0.219				

rabbit sera 3.50_3 and 3.48_1, respectively. On the other hand, in the equivalence zone and in the region of excess antibody (Tables II and III, and Table I, reference 1) the combining ratios of the components are approximately half again as high in Type III horse sera as in rabbit sera, and the difference may become even greater when antibody is present in large excess. In these regions of the reaction range a given amount of horse Type III pneumococcus anticarbohydrate precipitates less S III than does the same amount of rabbit antibody. In the administration of serum therapeutically this advantage of Type III rabbit serum[2] over horse serum would be lost as far as the

[2] Horsfall, Goodner, and MacLeod (10) have reported a larger number of protective units, also, per milligram, of antibody N in rabbit Type I antipneumococcus serum than in horse serum.

initial doses are concerned, since these would be given in the region of excess S III, in which the combining ratios are much the same.

The differences in combining ratios in the rabbit and horse antisera are paralleled by differences in the character and appearance of the precipitate. With antibody in large excess there are formed opaque floccules (Table II) which are easily broken up and resuspended. With less antibody in excess the precipitate becomes more and more compact, and at the S III excess end of the equivalence zone an almost transparent jelly is formed. This becomes entirely transparent when there is a greater excess of S III. In the horse antisera the precipitate remains flocculent throughout the entire range of antibody excess, becomes compact in the region of slight S III excess, and gradually turns more jelly-like and transparent as the inhibition zone is reached. The appearance of the precipitates, however, appears to depend on the N:S III ratio rather than on any species difference, since precipitates from the horse or rabbit with the same N:S III ratios are scarcely distinguishable.

The effect of increased salt concentrations in reducing the quantity of antibody precipitated by a given amount of S III is greater in the rabbit S III-antibody system than with the horse antibody (Table I, reference 11). Consequently, specific precipitates from rabbit antisera were found to give larger yields of highly purified antibody when dissociated with strong salt solutions (12).

In the experiments reported it has been shown that the behavior of Type III antipneumococcus rabbit sera over a large part of the precipitin reaction range may be described by means of the equations given with a degree of accuracy comparable with that found for the corresponding horse sera. The constants for these equations can be determined by the micro estimation of the amount of nitrogen specifically precipitable by a small number of properly chosen, accurately measured quantities of S III (*cf.* (1)). From the limited data at hand, summarized in Tables V and III, it would appear that the same relations apply to Type I antipneumococcus rabbit sera[3] in the only region investigated, that of excess antibody. Until the Type I pneumococcus specific polysaccharide used was prepared according to (5) it was found impossible to obtain accurate data as

[3] Also horse sera (unpublished data).

to the composition of the specific precipitate, since S and A were found together in the supernatants over a large part of the reaction range both in horse and rabbit antisera.

A notable difference between the S I-antibody system (Table V) and that of S III in both the horse and the rabbit sera is found in the far lower nitrogen to polysaccharide ratios in the Type I specific precipitate than in the Type III precipitate. The difference in the ratios in the horse and rabbit sera appears to be even greater in the Type I system than in that of Type III.

TABLE V

Addition of S I to Type I Antipneumococcus Serum 3.70

Amount S I added	Antibody N precipitated	Antibody N calculated from equation	Experimental ratio N precipitated to S I precipitated	Tests on supernatants
mg.	*mg.*	*mg.*		
0.025	0.139*	0.13	5.6	Excess A
0.050	0.226	0.23	4.5	Excess A
0.075	0.308	0.31	4.1	Excess A
0.100	0.371	0.37	3.7	Excess A
0.125	0.394	0.41	3.2	Excess A
0.150	0.431	0.44	2.9	No A or S
0.200	0.465			Excess S
0.250	0.480			Excess S
0.300	0.483			Excess S

Equation: Mg. antibody N pptd. $= 5.4\,S - 16.7\,S^2$. S max. = 0.162 mg.
A max. = 0.437 mg. N

Excess S I old (13) precipitated 0.08 mg. antibody N from this serum, S I acetyl (14) 0.204 mg. antibody N. With smaller amounts of S, the equivalence zone in both instances showed the presence of both A and S, instead of neither, as with S I prepared according to (5).

* S I nitrogen deducted from all values in this column.

While it is probable that the larger molecular weight of pneumococcus anticarbohydrate in the horse than in the rabbit (15, 16) is in part responsible for the higher nitrogen to polysaccharide ratios in specific precipitates from horse sera, the low ratios observed in both horse and rabbit Type I specific precipitates are not necessarily due to a higher molecular weight for the Type I specific polysaccharide (S I) than for S III. The relation between equivalence point ratio and molecular weight which Hooker and Boyd (17) sought to estab-

lish can scarcely apply to the specific polysaccharides. Indeed it is difficult to believe that the exceedingly viscous solutions yielded by the specific polysaccharides of pneumococcus as now prepared (5) are not the result of a relatively high molecular weight as well as due to the Coulomb forces (18) caused by the large number of acid groups in the molecules of substances such as S I and S III. The low molecular weights indicated some years ago (19) were found with the aid of material now known to have been partially degraded (5). Moreover, the assumption of a low polysaccharide molecular weight is not necessary for our quantitative theory of the precipitin reaction. According to current views as to polysaccharide structure, the pneumococcus specific polysaccharides, also, may be considered to have coil-shaped, zigzag, or rod- or thread-shaped molecules and to derive their immunological multivalence from recurrent chemical groupings characteristic for each pneumococcus type. With such a structure the factor determining combining ratios with antibody would not be the molecule as a whole, but the minimum chain length capable of reacting with a single molecule of antibody. This quantity may be calculated from the combining ratios in the region of extreme antibody excess, in which the polysaccharide may be considered to be "saturated" with antibody. Use is made in this calculation of the recent tentative assignment of molecular weights of about 150,000 and 500,000 to pneumococcus anticarbohydrate in the rabbit and horse, respectively (15).

For the three Type III antipneumococcus rabbit sera studied in detail, the mean value of 2R, the maximum calculated antibody N:S III ratio, was 13.5. It is seen that this number is between one-half and one-third the maximum calculated ratio (about 32, Table IX, reference 1) for horse sera under corresponding conditions. If these figures be multiplied by 6.3, the values 85 and 200 are obtained for 2R expressed as the antibody protein:S III ratio. Dividing these numbers into 150,000 and 500,000, the tentative rough estimates of the molecular weights of anticarbohydrate globulin produced in the rabbit and in the horse (15), respectively, one obtains 1800 and 2500 as the weight of the minimum chain lengths of S III reactive with an antibody molecule in each antiserum. Hypothetical units of this size would correspond to five to eight aldobionic acid groupings (20).

A similar calculation with the data at hand from only two sera with 2R = 5.4 and 14.4 for rabbit and horse Type I antipneumococcus sera, respectively, gives 4400 and 5500 for the minimum reactive chain lengths of S I, values about twice as great as those for S III. In accord with this, experiments with numerous Type I rabbit and horse antisera have shown that far more S I (5) is required to give maximum antibody precipitation than is necessary for the precipitation of antibody from Type III sera with S III. While any theoretical interpretation of these figures would be premature, it is at least evident from these values and the other ratios given that all through the reaction range, it requires roughly twice as much horse or rabbit antibody to combine with a given amount of S III as with the same weight of S I. Since in addition Type III pneumococcus produces far more S than does the Type I organism, both factors would probably contribute to the greater success of serum therapy in Type I pneumonia.

Equivalence zone ratios in the Type III rabbit antisera range from about 9 to about 6 at the two ends of the zone, corresponding roughly to one molecule of antibody for every 2600 to 3900 of polysaccharide weight units. In the inhibition zone, with much S III in excess, ratios of about 3 are indicated in the precipitate, corresponding to about 7900 units of S III per molecule of antibody. If the composition of the precipitate at the extreme antibody excess end of the range be represented by $\bar{S}A$, in which $\bar{S}$ represents the minimum combining weight of S III, and A a molecule of antibody, the composition of the precipitate in the equivalence zone would range roughly from $\bar{S}_3A_2$ to $\bar{S}_2A$, while in the inhibition zone $\bar{S}_4A$ would be reached. Since it has been shown (21) that the soluble compound formed in the inhibition zone contains one more unit of S III than the immediately preceding insoluble one, the composition of the soluble complex would approximate $\bar{S}_5A$.

Applying similar reasoning to horse anti-S III globulin, the equivalence zone ratios (Table VI, reference 1, and Table III) would correspond roughly to the limits of composition $\bar{S}_3A$ and $\bar{S}_6A$, while the inhibition zone complex would be of the order of $\bar{S}_{10}A$.

While these figures are only extremely rough approximations which will necessarily have to be revised, they at least give the order of magnitude of the equivalent combining ratios of the two components

of the specific precipitate at definite reference points or zones in the reaction range. The order of magnitude of the combining proportions appears sufficiently small to justify the classical chemical treatment given, although the tentative formulas are not necessarily those of single chemical components. Figures now available for antigen-antibody systems which have been studied will be reported in a subsequent communication.

SUMMARY

1. The reaction between the specific polysaccharide of Type III pneumococcus and homologous antibody in rabbit sera is quantitatively accounted for by expressions similar to those derived from the mass law for the corresponding horse sera. Preliminary data are also given for the Type I reaction.

2. Differences and similarities of the reaction with antibodies produced by the two animals are discussed.

3. Calculations are made of the equivalent composition of the specific precipitate at various reference points in the reaction range.

4. Certain theoretical and practical implications of the findings are pointed out.

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