CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION

II. The Identity of Precipitin and Agglutinin*

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Although the unitarian theory of antibodies has gained general acceptance since its formulation by Zinsser and others (1), quantitative evidence for the identity of agglutinins and precipitins has been lacking. Zinsser (2) has attempted to reconcile mathematically the apparent discrepancy between the serum dilutions in which agglutination and precipitation occur and Jones (3) has shown that collodion particles coated with egg albumin are agglutinated by small quantities of anti-egg albumin serum. Antibody concentrates, purified by Felton and Bailey (4), have been found to contain precipitins, agglutinins, opsonins, and protective antibody, but quantitative data were lacking until after the writers' preliminary report (5).

The development of quantitative micro methods for the estimation of precipitins (6, 7) and agglutinins (8) has now made it possible to investigate the quantitative correspondence of precipitin and agglutinin.

Pneumococcus Type I specific polysaccharide (acetyl form) (9),[1] a Chamberland filtrate of an autolyzed 8 day Type I Pneumococcus culture, Pneumococcus

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[1] A preparation of Type I pneumococcus specific polysaccharide in which the use of heat was avoided (9 *b*) precipitated the same amount of antibody from horse antibody solution B 78 as did the original broth filtrate or material prepared according to (9 *a*).

I S (Dawson M (10)) and R (Dawson S) strains, and Type I antipneumococcus sera were chosen as the objects of study. Serum H 701, which was a combined Pneumococcus I, II antiserum, was precipitated with a slight excess of Type II polysaccharide in order to leave only the Type I anticarbohydrate (*cf.* 8). To make the system approach as nearly as possible that of a single type specific hapten and its homologous antibody, the other sera were absorbed with C polysaccharide and protein derived from Type I R pneumococci. These sera still contained considerable amounts of group specific antibody (5), but it was found possible to prepare solutions containing antibody only to the type specific carbohydrate by repeated absorption with large amounts of Pneumococcus I R suspension.

Before using the Pneumococcus I R strain it was necessary to know whether it contained residual traces of type specific carbohydrate since it was to be used both for the determination of anti-R in the sera, and for the removal of the anti-R by absorption of the sera. That the method used was applicable was shown by a similar study of a Type III R strain, in which specific polysaccharide was actually present.

EXPERIMENTAL

The precipitin determinations were carried out by addition of known amounts of Type I pneumococcus specific polysaccharide to accurately measured quantities of absorbed serum or antibody solution and determination of the specifically precipitated nitrogen after 48 hours at 0° on the washed precipitates by the micro Kjeldahl method (6, 7, 11). Agglutinin determinations were made similarly (8), using measured volumes of washed pneumococcus suspensions and estimating the increase in nitrogen content. Heat-killed suspensions were used for all analyses, but formalinized suspensions were also used in absorbing sera. All determinations were run in duplicate at 0° for 48 hours in order to remove antibody as completely as possible (11).

1. Absence of Type Specific Polysaccharide in a Pneumococcus I R Strain.— The Pneumococcus I R strain used[2] (strain I-192 R (12)) had been degraded from a virulent Type I S strain (strain I-230 S) which was also used in all agglutinations with S organisms. It was assumed that if the I R suspension contained type specific substance it should remove all of the antibody from a type specific antiserum in a series of absorptions. The data in Table II on sera H 610, H 701, and antibody solution B 76 show that no antibody was taken out after the initial absorption. Since the limits of error of the quantitative agglutinin method are about ± 0.02 mg. of nitrogen and it was also found that 0.01 mg. of specific polysaccharide removed about 0.20 mg. of antibody nitrogen from antibody solution B 76, 0.02 mg. of antibody nitrogen would thus correspond to 0.001 mg. of specific polysaccharide. The amount of I R (Dawson S) suspen-

[2] Kindly supplied by Dr. Martin H. Dawson. The pneumococci used in this investigation were grown by Mr. C. M. Soo Hoo.

sion used was 2 ml., containing about 0.44 mg. of bacterial nitrogen or approximately 44 billion organisms.[3] This amount of suspension, therefore, contained no more than 0.001 mg. of specific polysaccharide. Similarly with a larger quantity of the same suspension and the stronger serum H 610, 0.66 mg. of bacterial nitrogen or approximately 66 billion organisms contained less than 0.001 mg. of type specific substance (Table I).

TABLE I

Absorption of Agglutinins from Horse and Rabbit Antisera with Pneumococcus I R, III R, and III S Suspensions

Absorption No.	1.0 ml. H 610 horse serum	4.5 ml. H 53300 horse serum	1.0 ml. R 348$_4$ rabbit serum		
	Agglutinin N removed by I R	Agglutinin N removed by III R*		Agglutinin N removed by	
				III R	III S
	mg.	*mg.*		*mg.*	*mg.*
1	0.16	0.14		0.098	0.176
2	0.00	0.08	Aliquot of 1st supernatant	0.07	0.022
3		0.06	Aliquot of 2nd supernatant	0.014	
4		0.06			
5		0.05	Total agglutinin N	0.182	0.198
6		0.05			
7		0.04			
		Absorption discontinued			

In absorptions 2 to 7 on serum H 53300 the entire supernatant from the preceding absorption was used.

* Centrifuged sediment from 2.0 ml. suspension. Agglutinin N determinations with III R and III S suspensions gave 0.56 and 0.54 mg. N per ml., respectively.

In order to remove the group specific antibody, 12 ml. of antibody solution B 76 were repeatedly absorbed with Pneumococcus I R organisms (about 3.5 mg. N per absorption). The pneumococci were first centrifuged, the supernatant was poured off, and the antibody solution was added to the bacterial sediment, taking care to obtain an even suspension. After 24 or 48 hours the

[3] The number of organisms was determined by comparison with a suspension of pneumococci standardized by counting. The numerical correspondence of bacterial nitrogen and the number of organisms is purely accidental.

agglutinated bacteria were centrifuged off and the supernatant was poured onto another portion of centrifuged organisms. In this way undue dilution was avoided. After about 12 absorptions, a determination of the anti-R on 1.0 ml. samples of the supernatant showed 0.016 mg. N per ml., and since this was within the limit of error of the determination (8) and equalled only 1.3 per cent of the type specific anticarbohydrate still present, absorption was discontinued. In the preparation of a large quantity of serum or of Felton antibody solution (13) free from group specific antibody it is convenient to divide the material into 12 to 15 ml. portions and absorb repeatedly with I R organisms as described for B 76.

2. Presence of Type Specific Polysaccharide in a Pneumococcus III R Strain.—In a similar manner it was found that a III R strain[2] (M III R (12)) actually contained a small amount of Type III specific polysaccharide. It will be seen from Table I that on repeated absorption of a Type III S antiserum, H 53300, with the suspension, small amounts of antibody nitrogen were continually removed and a limit was not reached. On determining the total agglutinin in a smaller amount of a weaker serum, R 348_4, with both Type III S and R suspensions (Table I), it was found that the same total amount of antibody nitrogen was ultimately removed by both strains. These results were interpreted on the basis that the Type III R strain contained a small amount of specific polysaccharide and this was confirmed as follows:

To 5.0 ml. of the bacterial suspension in saline 2.6 ml. of normal sodium hydroxide were added and the mixture was allowed to stand at 37° for 72 hours. The solution was neutralized to phenol red with hydrochloric acid, and was made up to 25.0 ml. and centrifuged free from traces of insoluble material. The salt concentration was then about 0.9 per cent. 3 ml. of this solution were set up with 1.0 ml. of a calibrated antibody solution (14) and the suspension was found to contain 0.0154 mg. of type specific polysaccharide per mg. of bacterial nitrogen—an appreciable amount.

Two different lots of the Type I S suspension used were found by this method to contain 0.462 and 0.493 mg. of type specific polysaccharide per mg. of bacterial nitrogen. The analyses were made as in the case of the Type III suspension except that the Type I polysaccharide used in calibrating the serum was treated with alkali at 37° for 72 hours in the same way as the bacterial suspension of which the polysaccharide content was to be determined, since Avery and Goebel (9) have shown that alkali treatment decreases the precipitating power of the polysaccharide.

3. Identity of Agglutinin and Precipitin.—In Table II are given the precipitin and agglutinin determinations. As will be noted, total agglutinin and total precipitin estimations were run, and in other instances a portion of the precipitin or agglutinin was removed and the agglutinin or precipitin in the supernatant was determined. In the last column are given the values for total antibody found, either for total agglutinin or precipitin alone, or for the sum of the analytical values for agglutinin plus precipitin. Serum H 610 and antibody solution B 76 were the same as those used in the first paper of this series (8), except that

TABLE II

Quantitative Comparison of Agglutinin and Precipitin in Type I Antipneumococcus Sera and Antibody Solutions

Laboratory designation of serum	Volume of serum or antibody solution used	Agglutinin N removed by Pn I	Strain used	Precipitin N found in supernatant	Remaining antibody N with Pn I S suspension	Total antibody N	Antibody N per ml.	Volume of serum or antibody solution used	Precipitin N removed	Agglutinin N in supernatant	Strain used	Remaining antibody N removed by Pn I S suspension	Total antibody N	Antibody N per ml.
	ml.	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*ml.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
Serum H 610	0.50	0.77*	I S	0.00	0.00	0.77	1.54	0.50	0.75†	0.05	I R		0.80	1.60
	0.50	0.32	I S	0.38	0.05	0.75	1.50	0.50	0.75†	0.02	I R	0.00	0.77	1.54
	0.50	0.25	I S	0.50	0.06	0.81	1.62	0.50	0.52††	0.26	I S		0.78	1.56
	0.50	0.16§	I R	0.59†	0.00	0.75	1.50	0.50	0.60	0.21	I S	0.01	0.82	1.64
Serum H 701 (1:1)**	1.00	1.16‖	I S	0.00	0.00	1.16	1.16	1.00	1.05	0.14	I S	0.00	1.19	1.19
	1.00	0.27	I S	0.73	0.16	1.16	1.16	1.00	1.05	0.13	I R	0.00	1.18	1.18
	1.00	0.33‖	I R	0.81†	0.03	1.17	1.17	1.00	0.89	0.31*	I S	0.00	1.20	1.20
Antibody B 76	0.50	0.82*	I S	0.00	0.00	0.82	1.64	0.50	0.75	0.01	I R	0.00	0.76	1.52
	0.50	0.53	I S	0.08	0.17	0.78	1.56	0.50	0.55	0.24	I S	0.00	0.79	1.58
	0.50	0.09§	I R	0.61†	0.07	0.77	1.54							
Antibody B 76 absorbed	0.50	0.61*	I S	0.00	0.00	0.61	1.22	0.50	0.52	0.10	I S	0.00	0.62	1.24
	0.50	0.16	I S	0.32	0.12*	0.60	1.20	0.50	0.54†	0.06	I S	0.00	0.60	1.20
	1.00	0.02	I R					0.50	0.35	0.25	I S	0.01	0.61	1.22
Antibody B 78 absorbed	1.00	0.69‖	I S	0.00		0.69	0.69	1.00	0.69†‡	0.05	I S	0.00	0.74	0.74
	1.00	0.30	I S	0.33	0.07	0.70	0.70	1.00	0.63	0.10	I S	0.00	0.73	0.73
								1.00	0.56	0.11	I S	0.02	0.69	0.69

* Two absorptions.

† Complete removal of anti-S as shown by excess S in supernatant.

†† 2 hours at 37°. Ice box overnight.

§ A second absorption with Pneumococcus I R removed no more antibody.

‖ Three absorptions.

** Contained 0.13 mg. anti-C N per ml.

‡ Broth culture filtrate of Pneumococcus I S used.

B 76 was somewhat more concentrated. "B 76 absorbed" and B 78 are the antibody solutions from which practically all of the group specific antibody was removed as described above. H 701 was a New York City Department of Health Type I, II mixed antipneumococcus serum from which the Type II antibody had been removed. The data for antibody solution B 75 have already been presented in the preliminary note (5) and are therefore not repeated.

The Type I specific polysaccharide used contained a small amount of C substance (15), so that I R agglutinin values were smaller after removal of precipitin.

In the experiments on the partial removal of agglutinin and precipitin, a known volume of bacterial suspension (or of polysaccharide solution) was added to a measured volume of serum or antibody solution, and after removal and analysis of the precipitate (6–8), an aliquot portion of the supernatant was set up with another measured volume of polysaccharide solution (or bacterial suspension). Finally, an aliquot of this second supernatant was set up with a known volume of Pneumococcus I S suspension in order to remove any traces of antibody remaining. The values in the table are corrected for the aliquots taken.

DISCUSSION

In order to determine whether or not precipitin and agglutinin are identical it was desirable to limit the investigation as nearly as possible to a single type specific antigen or hapten and its homologous antibody. However, pneumococci contain group specific antigens, and antipneumococcus sera may show varying amounts of group specific as well as type specific antibodies. It was therefore essential to have available a strain free from type specific antigen both for the purpose of estimating quantitatively the group specific antibody in a serum and for absorption of group specific antibody from such sera. Owing to the large number of absorptions necessary, the presence of even small amounts of type specific polysaccharide would result in removal of much of the antibody it was desired to retain.

The application of the quantitative agglutinin method (8) to establish the presence or absence of an antigenic component in the R strains used is illustrated in the section on experiments and summarized in Table I. It may be noted that Dawson (16) found it possible to cause reversion of the Pneumococcus M III R strain (which contains specific polysaccharide) to the S variant by *in vitro* as well as *in vivo* methods, whereas the I-192 R strain (which is free from specific polysaccharide) was exceedingly stable and could only be converted into the S form by subcutaneous injection with I S vaccine. The

analytical method given should therefore be of service in indicating the completeness of dissociation and possibly the stability of variants.

Data regarding the identity of agglutinins and precipitins are summarized in Table II. In the Type I antipneumococcus horse sera H 610 and H 701 it will be seen that type specific precipitin plus the small amount of residual group specific agglutinin equals total agglutinin, and that total antibody nitrogen remains the same when only a portion of the type specific antibody (anticarbohydrate) is first removed either as agglutinin or precipitin and the remainder as precipitin or agglutinin. Since the value for total antibody nitrogen in the combined precipitin-agglutinin estimations is the result of three independent sets of analyses, each involving several successive determinations, the precision attained is not as great as in the estimation solely of total agglutinin or total precipitin. The difference between the extreme values in the various sera ranges from 3.5 to 8.5 per cent of the total antibody content.

The data for sera H 610 and H 701 indicate the quantitative correspondence of the type specific anticarbohydrate agglutinin and precipitin within the limits of accuracy of the method. Using the mean values for total antibody nitrogen, antibody solution B 76, both unabsorbed and absorbed, yielded about 11.5 per cent more agglutinin nitrogen than precipitin nitrogen, while solution B 78 showed only a 4 per cent excess of type specific agglutinin. Since serum H 610, from which solution B 76 was prepared, did not show this effect, it would appear that a portion of the antibody had been slightly altered in the process of purification, a possibility already considered by Felton (13).

While the data for total type specific agglutinin and precipitin show the same quantity of each form of antibody it would still be possible that different substances, present in equal quantity, were involved. This possibility is eliminated, however, by the experiments on the partial removal of precipitin and agglutinin (Table II), since such a reduction of the content of precipitin or agglutinin resulted in an equal reduction of the amount of agglutinin or precipitin, respectively, remaining in the solution. Evidence is thus given for the identity of the type specific anticarbohydrate agglutinin and precipitin in these sera.

SUMMARY

1. The absolute, quantitative agglutinin method has been used for the determination of the presence or absence of small amounts of specific polysaccharide in pneumococcus variants.

2. A technique is described for the removal of group specific antibody from antipneumococcus horse serum.

3. The type specific anticarbohydrate agglutinin and precipitin are not only present in identical amounts in Type I antipneumococcus horse serum, but a reduction in one is also accompanied by a quantitatively identical reduction in the other, providing evidence for their actual identity. In purified antibody solutions somewhat more agglutinin than precipitin is found, possibly owing to alteration of a portion of the antibody in the process of purification.

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