CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION

III. A Reaction Mechanism and a Quantitative Theory*

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Recent and even current articles on specific bacterial agglutination reveal wide differences of opinion regarding the mechanism of the reaction and the factors influencing its course. Since much of this divergence appeared traceable to the essentially qualitative and relative nature of the methods used for the estimation of agglutinin titer, it was decided to attempt the assembly of more precise data on agglutination than had hitherto been available. As a first step an absolute, quantitative method was developed for the micro estimation of certain agglutinins (1), in which these are measured as the amount of antibody nitrogen actually combined with a given volume of bacterial suspension and in which an accuracy of 0.01 to 0.02 mg. of nitrogen is easily attainable. With this method and with the absolute, quantitative methods previously developed for the estimation of precipitins (2–4), it was possible to demonstrate the identity of agglutinin and precipitin in absorbed antisera containing only antibody to the type specific polysaccharide of Type I pneumococcus (5). It has now been possible to extend the analogy and to account quantitatively for this instance of specific bacterial agglutination by a purely chemical theory, much as had been accomplished in the case of the precipitin reaction (6).

EXPERIMENTAL

Heat-killed suspensions of Type I S (Dawson M) pneumococci were prepared as described in (1). It was found that on standing for some time appreciable amounts of polysaccharide appeared in the supernatants of even carefully washed

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suspensions, just as Craigie and Wishart (7) found antigen to leach out slowly from washed suspensions of the elementary bodies of vaccinia virus. In order to avoid confusion of the agglutinin reaction under investigation with a simultaneously occurring precipitin reaction, the suspensions were freshly centrifuged and washed once with saline before use. After this treatment only insignificant traces of specific polysaccharide remained in the supernatant.

Type I antipneumococcus horse sera H 701, H 702, and a Felton antibody solution B 78 were completely freed from group specific antibody by repeated absorption with I R (Dawson S) pneumococci as described in (5). Horse serum H 79 was absorbed with C substance (8) and protein prepared from a Type I R strain. Felton antibody solution B 75 was described in (1). The pH of antibody solution B 78 was 6.96, that of serum 701 was 7.34.

Estimation of the amount of S I[1] in the pneumococci was carried out as in (5) by dissolving a measured volume of the suspension in alkali, neutralizing after 72 hours at 37°C., and making up to a volume calculated to yield 0.9 per cent salt

TABLE I

Effect of Volume and Final Concentration of Antibody on the Amount of Antibody N in Agglutinated Pneumococci

Volume	Antibody N removed by 0.20 mg. bacterial N	Antibody N concentration in supernatant
ml.	*mg.*	*mg. per ml.*
4.0	0.33	0.15
8.0	0.34	0.074
12.0	0.33	0.050

concentration. Aliquot portions of this solution were analyzed according to (4) by means of an antibody solution calibrated with alkali-treated S I.

Agglutinin determinations were carried out by addition of accurately measured volumes of washed bacterial suspension to measured volumes of serum or antibody solution and estimation of the increase in nitrogen content. Heat-killed suspensions of Pn I were used. All determinations were run in duplicate unless otherwise stated and tubes were covered with tight fitting rubber caps.

Data on the effect of volume on the agglutination reaction in the region of excess antigen were included in (1). Additional data in the region of excess antibody are given in Table I. It will be seen that the amount of antibody nitrogen taken out by the bacteria after 48 hours at 0°, with occasional stirring, is independent of the volume just as in the precipitin reaction (6). Thus the agglutinin nitrogen

[1] This designation is used for the specific polysaccharide of Type I pneumococcus. Pneumococcus I suspensions are referred to as Pn I.

removed is independent of the concentration of antibody nitrogen in the supernatant.

In Table II are given data in the region of excess antibody on the effect of varying lengths of exposure of the Pn I suspensions to antibody. Suspension and serum were mixed in tubes and allowed to stand with occasional stirring. Increasing amounts of antibody nitrogen were taken up with increasing lengths of time.

The extremely slow rate under these conditions at 0° suggested the possibility of measuring the velocity of agglutination. 15 ml. of Pn I and 3 ml. of antibody

TABLE II

Effect of Time of Standing on Antibody N Taken up by Pn I at Various Temperatures

Time	Felton antibody solution B 75	Serum H 79			
	0°	0°	25°	37°	55°*
hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1					0.46†
2				0.37	
6					0.53
19	0.33	0.27	0.36	0.44	
24					0.58
43			0.39		
48				0.44	0.58
72	0.40				
days					
5			0.43		
10				0.52	
12		0.34			

* Control tubes containing serum alone tended to gel at this temperature unless diluted with saline.

† One determination.

solution B 78 were added to 32 ml. of saline and the mixture was stirred mechanically in an ice bath. The temperature remained constant at +0.5°C. 5 ml. samples were withdrawn at varying intervals and pipetted into 5 ml. of cold saline and centrifuged at about 3000 R.P.M. in a refrigerated centrifuge.[2] The precipitates were washed and analyzed in the usual manner. It will be seen from Table III that with efficient stirring the reaction is an extremely rapid one, being more than 80 per cent complete in 5 minutes. Maximum combination of antibody requires a considerably longer period. Even when this is attained the aggregates

[2] Manufactured by the International Equipment Company, Boston.

formed are much smaller than in the incomplete reaction under the usual conditions.

In accordance with the above experiments it was found possible to use the usual arrangement of tubes and take out the maximum antibody nitrogen for any given set of conditions if the mixtures were shaken on a shaking machine oscillating with sufficient rapidity to keep the Pn I in suspension. A machine of the type[2] adopted for streptolysin determinations proved adequate. Shaking at 37° was carried out in an incubator, and at 0° the tube racks were placed in a box, packed with crushed ice, and shaken, the ice being replenished as often as necessary. Table IV shows the result of shaking for varying lengths of time. Values in parentheses were obtained by use of a mechanical stirrer instead of shaking. It will be seen that within the limit of error of the determinations stirring and shaking yielded identical results. Since the dilution in the stirred mixtures was more than two and one-half times as great, this confirms the finding (Table I) that antibody N removed is independent of the concentration. Two different lots

TABLE III

Velocity of Combination of Antibody and Pn I

Time	Antibody N removed	Time	Antibody N removed
min.	*mg.*	*min.*	*mg.*
5	0.12	60	0.15
15	0.14	90	0.16
25	0.14	120	0.16
40	0.14	150	0.16

Samples taken contained 0.25 mg. bacterial N.

of Pn I were used for the 37° and 0° experiments with B 78, but for sera H 701 and H 702 the same suspension was used for all determinations and results at different temperatures and salt concentrations are strictly comparable. It will be observed that in the region of excess antibody much more agglutinin N was taken out by the same amount of suspension at 37° than at 0°. This will be discussed below.

After determination of the time necessary to reach equilibrium, a series of tubes containing 1.0 ml. of serum or antibody solution and increasing amounts of freshly washed Pn I was set up in duplicate and shaken at the desired temperature until equilibrium was reached. The tubes were then centrifuged at the same temperature and the precipitates washed twice in the cold (1) and analyzed for nitrogen. Agglutinin N was found by subtracting the bacterial N. At the same time an accurately measured portion of Pn I was dissolved in alkali and the S content determined as described above. In the experiments in which the final salt concentration was to be 2 M, a volume of 3.85 M salt equal to the volume of serum and suspension used was first added. Table V shows the results obtained

TABLE IV

Antibody N Removed from Antipneumococcus I Solution and Sera by Pn I on Shaking for Varying Periods

Time	B 78		H 701				H 702			
	0.9 per cent NaCl		0.9 per cent (0.15 M) NaCl		2 M NaCl		0.9 per cent (0.15 M) NaCl		2 M NaCl	
	37°	0°	37°	0°	37°	0°	37°	0°	37°	0°
hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.5	0.22									
1		0.22			0.24					
1.5	0.23*									
2		0.23	0.31	0.13	0.24*	0.15	0.27	0.16	0.25	0.17
3	0.23	0.26*			0.23					
4			0.35	0.14	0.23 (0.22)	0.15	0.28	0.17	0.25	0.19
5		0.25								
6	0.23		0.35 (0.32)	0.14 (0.13)		0.16	0.29† (0.25)	0.17 (0.21)	0.25 (0.23)	0.20 (0.19)
7.5						0.16* (0.15)				
Bacterial N used	0.35	0.34	0.12	0.09	0.11	0.09‡	0.16	0.16	0.16	0.16

Values in parentheses obtained with mechanical stirring instead of shaking. Aliquot portions analyzed in triplicate.

* One determination only.

† A duplicate set of tubes, after 6 hours at 37°, was shaken 8 hours at 0°, yielding 0.32 mg. of antibody N.

‡ Bacterial N of the suspension in 0.9 per cent NaCl was used, owing to the difficulty of centrifuging non-agglutinated Pn I in 2 M NaCl.

TABLE V

Addition of Increasing Amounts of Pneumococcus I S (M) Suspension to 1 Ml. of Serum or Antibody Solution at Various Temperatures and Salt Concentrations

Bacterial N	Equivalent S I content	Total N precipitated	Antibody N precipitated	Ratio N:S in precipitate	Antibody N calculated from equation	Total N precipitated	Antibody N precipitated	Ratio N:S in precipitate	Antibody N calculated from equation
mg.	mg.	mg.	mg.		mg.	mg.	mg.		mg.
		Antibody Solution B 78, 0.15 M Salt, at 37°				B 78, 0.15 M Salt, at 0°			
0.122	0.017	0.255	0.17	7.1*		0.218†	0.07	4.1*	
0.244	0.034	0.494	0.24	7.1	0.24	0.454	0.17	5.0*	
0.366	0.051	0.718	0.34	6.7	0.34	0.642	0.24	4.7*	
0.488	0.068	0.916	0.41	6.0	0.42	0.852	0.33	4.9	0.33
0.732	0.102	1.302	0.56	5.5	0.54	1.216	0.45	4.4	0.46
0.976	0.136	1.576†	0.59	4.3	0.59	1.574	0.56	4.1	0.56
1.220	0.170	1.840	0.61	3.6		1.880	0.62	3.6	0.63
1.464	0.204	2.076	0.60	2.9*		2.188	0.69	3.4	0.68
Serum, salt		0.014				0.036			
		mg. antibody N pptd. = 8.0 S − 26.9 S²				mg. antibody N pptd. = 5.7 S − 11.6 S²			
		S max. = 0.149 N max. = 0.594 calcd.				S max. = 0.246 N max. = 0.70 calcd.			
		0.61 found				0.69 found			
		Serum H 701, 0.15 M Salt, at 37°				H 701, 0.15 M Salt, at 0°			
0.064	0.0165	0.254	0.18	10.9*		0.187	0.12	7.3*	
0.096	0.025	0.369	0.27	10.8	0.28	0.290	0.19	7.6*	
0.127	0.033	0.476	0.34	10.3	0.36	0.376	0.25	7.6*	
0.191	0.0495	0.686	0.49	9.9	0.49	0.534	0.34	6.9*	
0.255	0.066	0.868	0.61	9.2	0.59	0.722	0.47	7.1	0.47
0.382	0.099	1.060	0.67	6.8	0.72	1.030	0.65	6.6	0.63
0.509	0.132	1.202	0.69	5.2		1.244	0.74	5.6	0.74
0.637	0.165	1.340	0.70	4.2*		1.414	0.78	4.7	0.79
Serum, salt		0.008				0.000			
		mg. antibody N pptd. = 12.5 S − 53.2 S²				mg. antibody N pptd. = 8.8 S − 24.5 S²			
		S max. = 0.1175 N max. = 0.734 calcd.				S max. = 0.180 N max. = 0.79 calcd.			
		0.70 found				0.78 found			

		H 701, 2 M Salt, at 37°				H 701, 2 M Salt, at 0°			
0.064	0.0165	0.222	0.16	9.7	0.15	0.208	0.14	8.5*	
0.096	0.025	0.290	0.19	7.6	0.20	0.304	0.21	8.4	0.21
0.127	0.033	0.354	0.23	7.0	0.24	0.394	0.27	8.2	0.26
0.191	0.0495	0.454	0.26	5.3	0.28	0.534	0.34	6.9	0.34
0.255	0.066	0.540	0.29	4.4		0.638	0.38	5.8	0.39
0.382	0.099	0.688	0.31	3.1*		0.796	0.41	4.1	
0.509	0.132	0.828	0.32	2.4*		0.952	0.44	3.3*	
0.637	0.165	0.980	0.34	2.1*		1.120	0.48	2.9*	
Serum, salt	0.000					0.000			
		mg. antibody N pptd. = 10.6 S − 100 S³				mg. antibody N pptd. = 9.9 S − 59.8 S³			
		S max. = 0.053 N max. = 0.28 calcd.				S max. = 0.083 N max. = 0.41 calcd.			
		0.34 found				0.48 found			

For serum H 702 the equations were:

0.15 M salt: At 37°, mg. antibody N pptd. = 6.9 S − 16.5 S³	At 0°, mg. antibody N pptd. = 5.8 S − 11 S²‡
S max. = 0.209 N max. = 0.72 calcd.	S max. = 0.263 N max. = 0.76 calcd.
0.74 found	0.83 found
2 M salt: At 37°, mg. antibody N pptd. = 7.7 S − 44.8 S³	At 0°, mg. antibody N pptd. = 6.3 S − 20.7 S³
S max. = 0.086 N max. = 0.33 calcd.	S max. = 0.152 N max. = 0.48 calcd.
0.40 found	0.53 found

* Points not considered in calculating equation.

† One determination discarded.

‡ Only two points not at maximum ratio available for calculation.

in these experiments. The ratios given were calculated from the determinations of antibody N in the agglutinated Pn I and the S I content of the Pn I suspension used, although it is by no means certain that all of the S I in the suspension is capable of reacting. If it is assumed that the same proportion of the S I reacts at a given temperature throughout the entire reaction range, the observed N:S ratios would then be divided by the same factor and the character of the results would be unchanged. It will be seen from Table V that the ratios reach an upper limit with decreasing amounts of suspension. This was also determined by the

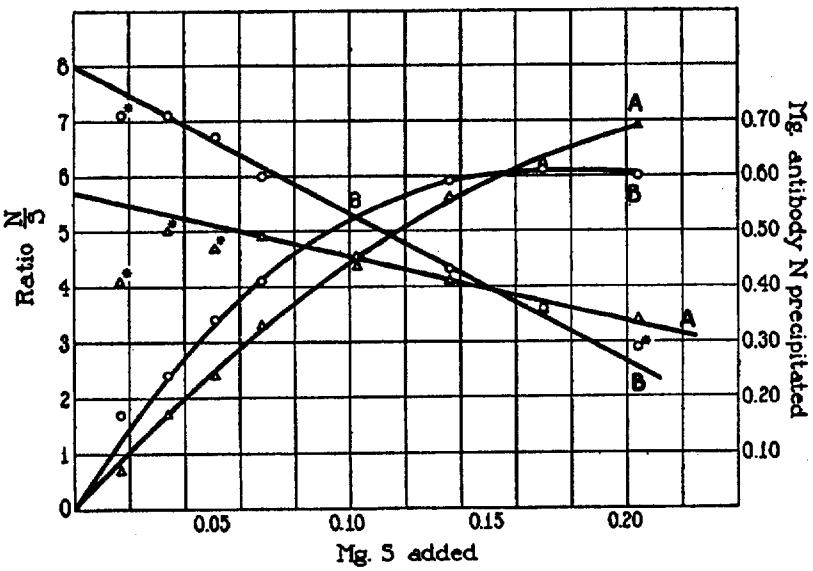


FIG. 1

addition of a constant amount of Pn I to increasing quantities of serum H 79 at 37°.

Volume of serum, *ml.*.....	0.5	1.0	1.5	2.0	2.5	3.0
Antibody N removed, *mg.*.	0.47	0.53	0.60	0.63	0.62	0.63

The amount of antibody N removed by 0.26 mg. of bacterial N reached a definite limit as the volume of serum added was increased.

In the region in which a change of ratio is observed the equation

$$N = 2RS - \frac{R^2}{A} S^2$$

derived for the precipitin reaction (6) was found to hold. In Table V are given the equations calculated from the data at each temperature and salt concentration. The antibody N values found are also compared with those calculated from the above equation. Fig. 1 shows the curves obtained in the case of antibody solution B 78 at 0° (curve A) and at 37° (curve B) in 0.9 per cent saline by plotting antibody N in the agglutinated Pn I against the polysaccharide content of the Pn I. Lines satisfying the equation

$$\frac{N}{S} = 2R - \frac{R^2}{A} S$$

were obtained by plotting against S I the ratios of agglutinin N taken out to S I in the Pn I used (Fig. 1, lines A and B). The method of least squares was used in

TABLE VI

Comparison of Maximum Calculated and Found Antibody N:S I Ratios

Serum	Maximum N:S I ratio from equation	Maximum N:S I ratio found	Calculated ratio / Found ratio
701, 0.15 M salt, 37°	12.5	10.0	1.25
" " " " 0°	8.8	7.4*	1.19
" 2 " " 37°	10.6	9.7	1.09
" " " " 0°	9.9	8.5	1.17
B 78, 0.15 " " 37°	8.0	7.1	1.13
" " " " 0°	5.7	4.6*	1.24
702, 0.15 " " 37°	6.9	5.4*	1.28
" " " " 0°	5.8	4.2*	1.38
" 2 " " 37°	7.7	6.7	1.15
" " " " 0°	6.3	5.6*	1.12

* Mean of maximum ratios determined experimentally.

every case to calculate the line best fitting the data. Points in the region of excess antigen, as well as those beyond the first point with maximum ratio, were not used in the calculations. These points are marked with asterisks in Table V and Fig. 1. The data obtained in 2 M salt solution are also in agreement with corresponding results for the precipitin reaction (9). Similar data were obtained with serum H 702 in 0.15 and 2 M salt solution at both temperatures.

A comparison of the maximum calculated and found ratios of antibody N to S is made in Table VI. It will be seen that the theoretical maximum ratio, 2 R, (*i.e.*, the intercept of the line on the *y* axis) is approximately equal to 1.2 times the experimentally determined maximum ratio.

It will be noted from Table V and Fig. 1 that throughout the region of antibody excess a given amount of Pn I removes more antibody N at 37° than it does when set up at 0° with serum in the same proportions. The following experiment was

performed in an effort to explain this effect. Four tubes containing 1.0 ml. of Pn I suspension and 1.0 ml. of serum were shaken for 6 hours at 37°. One pair was then shaken at 0° for 8 hours. Agglutinin N was determined in both sets of tubes. It was found that more agglutinin N was removed by shaking at 37° and then at 0° than was taken out at 37° alone (see footnote, Table IV). The difference in N removed at 37° and at 0° is very markedly reduced in 2 M salt.

As in the precipitin reaction it was not found possible to take out at 0° antibody remaining after the maximum antibody had been taken out at 37° with excess Pn I.

In the region of excess antibody, the agglutinated bacteria are very easily and uniformly resuspended in saline. In the region of excess polysaccharide, as in the precipitin reaction, large clumps are formed which cannot be easily dispersed.

To determine whether or not Pn I, agglutinated in the region of excess antibody, is capable of combining with more Pn I, the following experiment was set up.

To each of a series of tubes were added 0.5 ml. of Pn I S suspension (0.19 mg. N) and 1.5 ml. of serum, a large excess. The agglutinated bacteria were allowed to stand in the ice box for 48 hours and were centrifuged and washed twice with saline. The second washing was set up with 0.25 ml. of fresh Pn I suspension and failed to show agglutination, indicating that all uncombined antibody had been washed out. The original agglutinated (sensitized) Pn I was then resuspended uniformly in saline, and 0.25 ml. portions of various pneumococcus suspensions or measured amounts of specific polysaccharide (S I) were added. The results are shown in Table VII.

Eagle, Smith, and Vickers (10) have found that the partial coupling of pneumococcus Type I antibody with diazotized sulfanilic acid prevented the antibody from precipitating S I although it still gave definite agglutination with Pn I. It was concluded that S I failed to combine with the azo antibody since addition of untreated Type I antipneumococcus serum gave a precipitate. However, agglutination would also fail to occur if the polysaccharide no longer combined with antibody, since it has been shown that Type I anticarbohydrate precipitin and agglutinin are identical (5). If, on the other hand, soluble compounds of S I and azo antibody were formed, such compounds should be as readily capable of reacting with unaltered antibody to form precipitates as is uncombined S I. It has long been known that polysaccharide and antibody can combine in proportions varying over a wide range (11). Eagle, Smith, and Vickers' experiment was accordingly repeated with additional controls.

6 ml. of Type I antipneumococcus horse serum were coupled with 6 ml. of diazotized sulfanilic acid as described in (10). 2 ml. of the resulting azo antibody, freed from excess diazosulfanilic acid, failed to precipitate 0.05 mg. of S I or its deacetylated degradation product, but agglutinated 2 ml. of Pn I suspension (0.2 mg. N per ml.). 1 ml. of Type I antiserum was added to the tubes containing S I, resulting in immediate precipitation. As an additional control, an egg albumin-anti-egg albumin precipitate was formed in 2 ml. of the azo antibody solution by addition of 0.34 mg. of egg albumin and 0.5 ml. of a potent anti-egg albumin serum. All tubes were centrifuged and washed in the cold with saline until the washings were colorless. The egg albumin-anti-egg albumin precipitate was white, while the precipitates in the other tubes were definitely yellow. S I had therefore combined specifically with the azo antibody, not only on the surface of Pn I, where agglutination occurred, but in solution as well.

DISCUSSION

The mechanism of specific bacterial agglutination (reviewed in (12, 13, 14)) has been considered either as an adsorption process or as a precipitin reaction taking place at the cell surface (15). Although adsorption is now generally considered in its chemical aspects, Ivanovics (16) as late as 1935 chose to interpret bacterial agglutination as an example of physical adsorption. This conclusion is open to criticism on account of the large error of the experimental method used, the failure to eliminate simultaneously occurring precipitin reactions, and the complexity of the antigen-antibody system studied.

From the data given in Table I and reference 1 it is evident that a simple treatment according to classical chemical laws is as inadequate in accounting quantitatively for specific bacterial agglutination as is the Freundlich adsorption isotherm, since both depend on the concentrations of the reactants at equilibrium. As in the precipitin reaction (6) the composition of the agglutinated mass depends rather on the proportions in which the components are mixed (Table V).

In order that figures such as those given in Table V should be of significance it was necessary first to find the conditions under which the maximum amount of antibody nitrogen would be taken out by Pn I throughout the reaction range. It was observed that removal of antibody was too slow when tubes were mixed only at intervals (Table II), although this procedure was satisfactory in the precipitin reaction. This was due not to a slower rate of combination of polysaccharide and antibody in agglutination, but more probably to the fewer collisions between Pn I and antibody molecules. Mechanical stirring (Table

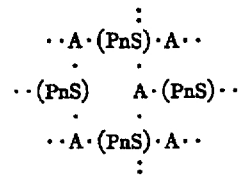
III) resulted in rapid attainment of maximum combination, as did also the use of a shaking machine (Table IV). A technique was thus available for a study of the agglutination reaction similar to that made of the precipitin reaction.

The data so obtained (Table V) indicate that equations of the same form as were valid for the precipitin reaction (6) serve also to describe quantitatively the agglutination reaction between Pn I and homologous type-specific anticarbohydrate. In the derivation of these equations it was assumed that S (polysaccharide) and A (antibody) first combine in a bimolecular reaction to yield AS, followed by the competing bimolecular reactions (in the region of excess antibody),

$$AS + A \rightarrow AS \cdot A$$
$$AS + AS \rightarrow AS \cdot AS$$

and that reactions of this type continue with increasing complexity until aggregates are formed which are large enough to separate from solution. In the derivation, also, it was found that volume factors cancelled, so that the end-result was independent of the final concentrations of the components. Since this condition is also shown experimentally to obtain in the agglutination reaction studied (Table I), and since a similar type of equation is found to apply to this reaction (Table V) as well as to specific precipitation, it would appear justifiable to consider this instance of specific bacterial agglutination as a precipitin reaction at the bacterial surface,[3] the more so as the same substances enter into both reactions (5).

According to this mechanism the mass of agglutinated Pn I would consist of pneumococci held together by combination of S I on the pneumococcus surface with antibody molecules. This three-dimensional process might be represented two-dimensionally as follows:



[3] In this discussion "bacterial surface" is defined as the portion of the Pn I cell to which reactive S I molecules are attached. This would not necessarily coincide with the external surface.

in which (PnS) represents pneumococcus with an unknown number of molecules of S at the reactive surface or surfaces. A somewhat similar qualitative representation of agglutination has been given by Marrack (14).

Agglutination of Pn I differs in at least three respects from the precipitin reaction. It will be noted from Table V that the antibody N:S I ratios reach a maximum in the region of excess antibody, beyond which more antibody cannot be forced into reaction. The value of 2 R given by the equation is usually about 1.2 times the experimentally determined maximum, as shown in Table VI. A possible explanation would be that antibody molecules, when present in large excess, are prevented by spatial exigencies from combining with all of the immunologically reactive S groupings available. It appears reasonable to assume that this crowding would occur at lower N:S I ratios at the Pn I surface than in the precipitin reaction, in which S I is in solution.

Another difference from the precipitin reaction is in the apparently lower combining ratios of the components. Since it is not known whether or not all of the S found in Pn I is available for reaction, as is assumed in calculating the ratios given, these values have only a relative significance. If, under a given set of conditions, the same fraction of S I reacts throughout the whole range, the entire set of ratios could be multiplied by a factor inversely proportional to the fraction reacting.

A third difference is that throughout the antibody excess range more antibody is taken out at 37° than at 0° (Table V and Fig. 1). An even larger amount of antibody N is removed by the same quantity of Pn I when the reaction is carried out first at 37° and then at 0° (footnote, Table IV). It would seem that a larger proportion of the S I present is capable of reacting at 37° than at 0°, but when this has once entered into reaction, continuation of the process at 0° takes place much as in the precipitin reaction, with more antibody removed at the lower temperature. Possibly the effect noted is due to swelling of the Pn I at 37°, resulting in the exposure of more of the S I content.

The differences between the precipitin and agglutinin reactions are thus seen to be mainly mechanical, rather than differences in principle, and to be conditioned by the spatial limitations of a chemical reaction taking place, in agglutination, at the bacterial surface. Since con-

siderable confusion would be caused by the adoption of conclusions of Eagle, Smith, and Vickers (10) indicating a fundamental difference between agglutination and precipitation, it is necessary to mention here that these conclusions are easily shown to be based on insufficient evidence. By the repetition of their experiments with the necessary controls (page 895) it has been shown that Type I pneumococcus anticarbohydrate which has been coupled with diazotized sulfanilic acid actually combines with S I, although it does not yield a precipitate. It is therefore not surprising, in view of the existing knowledge of the precipitin reaction and the demonstrated identity of anticarbohydrate precipitin and agglutinin (5) that agglutination of Pn I is effected by the same azo antibody which merely combines with S I in solution to give soluble compounds.

According to the new quantitative theory the entire process of specific bacterial agglutination may be most simply treated as a continuous progression of competing bimolecular chemical reactions. The theory makes no distinction between the initial combination of antigen with antibody and the subsequent aggregation, the latter being considered as a continuation of the process of combination of multivalent antigen with multivalent antibody until aggregates are formed which are large enough to flocculate.

While the presence of salts is necessary for flocculation, and their function is discussed in greater detail below, it is believed that the experiments recorded in Table VII indicate the essentially chemical nature of even the flocculation stage of specific bacterial agglutination. By interruption of the process of specific bacterial agglutination at a definite stage it was found possible to study the specificity of further particulation under constant conditions of salt concentration and to test predictions based on the theory.

Pn I cells, agglutinated with a considerable excess of antiserum, were washed with saline until the supernatant contained no agglutinin. The agglutinated (sensitized) Pn I cells were then evenly resuspended in saline and divided into several portions. According to the new quantitative theory, Pn I, agglutinated in the region of excess antibody, would still have available on the surface of the particles some of the specifically reactive groupings of the originally multivalent antibody. These particles, then, should be able to combine with S I on

the surface of freshly added unsensitized Pn I, and reagglutination should take place to form larger aggregates. It will readily be seen from Table VII that this prediction was verified, and that the effect is specific, since it is not given by Pn II or III, or by Pn I R (Dawson S) cells under identical conditions of salt concentration and, in the

TABLE VII

Addition of Pneumococcus or S I to Washed, Resuspended, Agglutinated Pn I S (M)

Material added	Result
Unsensitized Pn I S (M) suspension	Rapid reagglutination into large clumps, clear supernatant
" Pn I R suspension	No visible reaction
Pn I S supernatant*	" " "
Unsensitized Pn III S (M) suspension	" " "
" Pn II S (M) suspension	" " " †
1 mg. S I (Pn I specific polysaccharide)	" " "
0.10 mg. S I	Reagglutination‡
0.01 mg. S I	" ‡
0.001 mg. S I	Partial reagglutination
0.0001 mg. S I	" "
0.10 mg. S II	No visible reaction
0.9 per cent saline	" " "

Reagglutination failed to take place if sensitized Pn I and fresh Pn I were washed with 5 per cent glucose until supernatants no longer reacted for Cl^-. Addition of NaCl caused immediate flocking. The function of electrolyte is discussed in the text.

* Freshly washed, unsensitized Pn I S suspension was again centrifuged and 0.25 ml. supernatant was added to determine whether effect was due to any S I which might be dissolved from bacteria.

† Pn I S suspension was next added. Immediate flocculation took place. The large clumps formed settled rapidly. Type II serum added to the turbid supernatant caused immediate agglutination.

‡ The contents of these tubes formed large clumps, resembling those with Pn I suspension.

case of Pn II and III, presumably similar conditions of potential and cohesive force. Reagglutination is, moreover, produced almost as completely by suitable amounts of S I in solution,[4] so that the conclusion seems inescapable that particulation, as well as the original anti-

[4] And not by S II, the specific polysaccharide of Pn II.

gen-antibody combination, is a chemical process. The quantitative data given indicate that the particulation is a result of the original bimolecular combination as continued in a series of competing bimolecular reactions, much as in the precipitin reaction (6). It is also shown in Table VII that most of the Pn II added fails to participate in the reagglutination when Pn I is subsequently added, but may be agglutinated by pouring off the turbid supernatant and adding Type II antiserum. A similar separate agglutination of mixed microorganisms was observed by Topley, Wilson, and Duncan (17), who also concluded that the particulation phase of agglutination was strictly chemical, in confirmation of Marrack's views (14).

The results of all of these experiments, in accord with the new quantitative theory, indicate the primary importance of the chemical interaction of multivalent antigen with multivalent antibody in completing, as well as initiating, the process which the bacteriologist calls specific bacterial agglutination. If this be true, specific bacterial agglutination differs fundamentally from other instances of agglomeration and agglutination of suspended particles, and the analogy with these, so often cited, does not apply. The function of the electrolyte in specific bacterial agglutination would seem to be the secondary one of providing ions for the ionized salt complexes in which form antibody probably reacts (18), and in addition, of minimizing electrostatic effects due to the presence of many ionized groupings on the particles, effects which might interfere with the completion of particulation by chemical interaction.

Whether or not the initial bimolecular antigen-antibody reaction on the bacterial surface can take place in the absence of electrolyte, the reactants carry ionized groups and it is evident that the succeeding competing bimolecular interactions between polysaccharide molecules on partly sensitized cells and additional antibody in solution or on other cells would soon result in the formation, in the absence of electrolyte, of particles carrying large numbers of ionized groups. Coulomb forces on such particles are known to cause abnormally great viscosities and Donnan effects, so that it would not be surprising if these forces would prevent the continuation of the chemical reactions resulting in the completion of what is commonly recognized as specific bacterial agglutination. The effect of removal of salts is shown at the bottom

of Table VII. Only when the effect of these forces is eliminated by a sufficient ionic atmosphere, on addition of electrolyte, is it possible to obtain significant figures for viscosity, osmotic pressure, sedimentation constants, and the like. To ascribe a similar rôle to electrolytes in specific bacterial agglutination would seem reasonable and consistent, for after reduction of the Coulomb forces the growing particles could again interact chemically, and the process of agglutination be completed.

This conception of the effect of electrolytes is in part based on the charge-reducing properties of salts, like the older views of Bordet (19), Northrop and De Kruif (20), Shibley (21), and others, but involves a shift of emphasis in that, regardless of any such reduction, specific agglutination takes place only when the primary chemical interaction between multivalent antigen and antibody can proceed toward completion. Many irregularities and inconsistencies in the relation of physical forces to agglutination are thus eliminated and explained.

SUMMARY

1. By the application of an absolute, quantitative microchemical method for the estimation of agglutinins, precise data have been obtained on the course of the agglutination of Type I pneumococcus by homologous anticarbohydrate.

2. Within the limitations imposed by the necessity for the agglutination reaction to take place at the bacterial surface, the reaction is shown to be analogous to the precipitin reaction and subject to the same laws.

3. The entire process of a typical instance of specific bacterial agglutination has been quantitatively accounted for on a purely chemical basis and expressed in the form of equations derived from the law of mass action.

4. Experimental verification of predictions based on the theory has shown a fundamental difference between this instance of specific bacterial agglutination and the commonly adduced analogies, and necessitated a revision of current conceptions regarding the rôle of electrolytes and of physical forces in the reaction.

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