QUANTITATIVE CHEMICAL STUDIES ON COMPLEMENT OR ALEXIN

II. The Interrelation of Complement with Antigen-Antibody Compounds and with Sensitized Red Cells*

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In the preceding paper (1) a method was described by which complement, or that portion of it which combines with antigen-antibody precipitates, could be measured in weight units; that is, in absolute rather than relative terms. It was estimated that the samples of pooled guinea pig sera analyzed contained from 0.04 to 0.06 mg. of complement nitrogen per ml., corresponding to 0.25 to 0.4 mg. of complement combining component, if the substance is a globulin, as seems certain (2–4). With the aid of these figures it is now possible to define quantitative relations, under the conditions used, between complement, antigen, antibody including hemolysin, and the sensitized sheep red cell.

It has long been known that titration of varying amounts of hemolysin against varying quantities of complement yields dilution end-point curves roughly of parabolic form. This renders difficult the establishment of definite "units" and the determination of the minimal amounts of antigen, antibody, hemolysin, and complement effective in complement fixation tests. Full data are therefore given below, and from these it should be possible to derive any conventional "unit" desired whether it be based on initial, 50 per cent, complete hemolysis, or some other factor. While such "units" have hitherto been of great practical value they are not essential to a consideration of the relations between the reacting components in complement fixation and are therefore not used in the present discussion.

EXPERIMENTAL

The Relation between Complement, Hemolysin, and Red Cells.—The rabbit anti-sheep cell hemolysin used in Experiments 1, 2, and 3 of the preceding

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paper (1) contained 0.37 mg. of hemolysin N per ml.[1] In each instance an equal volume of 1:1250 hemolysin dilution was added to a 5 per cent sheep red cell suspension. 0.2 ml. portions of sensitized cell suspension were used to determine the smallest amount of guinea pig serum permitting complete hemolysis. The data obtained, including cell counts[2] and derived calculations, are summarized in Table I.

The Relation between Complement, Antigen, and Antibody.—

Experiment 10.—Duration 5 hours. Dilutions were made with 0.85 per cent saline and the same pipette was used for corresponding dilutions of each reagent. The anti-egg albumin (Ea) rabbit serum and the antipneumococcus Type III rabbit serum were those used in the preceding paper and contained 2.3 mg. of anti-Ea N and 7.0 mg. of anticarbohydrate N, per ml., respectively.

Titration of Complement and Hemolysin.—To a mixture of rabbit anti-sheep cell hemolysin (in decreasing amounts; volume, 0.3 ml.) and diluted complement (volume, 0.1 ml.) 0.1 ml. of a 4 per cent suspension of washed sheep red cells was added and the mixture incubated at 37°C. for 45 minutes. Values at the heads of the columns in Table II refer to the actual amount of undiluted hemolysin or complement present in a given set of tubes.

For purposes of calculation the average value, 0.05 mg., or 50 γ of complement combining component (C'1) N per ml., will be used. The hemolysin contained 0.50 mg. of antibody N per ml. reactive with washed sheep cell stromata[1] and this will be taken as a first approximation to the actual hemolysin N content, although it is possible that not all of the antibody is effective as hemolysin. It is evident from Table II that cells sensitized with 0.008 γ of hemolysin N required the presence of approximately 0.3 γ of C'1 N for complete hemolysis (column 4), while the same quantity of cells sensitized with 0.02 γ of hemolysin N required only 0.13 γ of C'1 N for complete hemolysis. In two other similar titrations complete hemolysis resulted with as little as 0.08 γ of C'1 N, in one series only in the tube containing 0.04 γ of hemolysin N, and not in those with more or less antibody. In the other series complete hemolysis occurred in the tubes containing from 0.02 to 0.06 γ of hemolysin N, the largest amount used.

After titration of hemolysin and complement 0.1 ml. portions of antipneumococcus Type III rabbit serum dilution containing the amount of antibody N indicated in column 1 of Table III were mixed with 0.1 ml. of complement dilution containing the volume of guinea pig serum shown in the other column headings. To these mixtures were added 0.1 ml. portions of S III[3] dilution containing the quantity of S III given for each section of Table III. If, as was usual at high dilutions, precipitation did not occur before addition of complement the order in which the reagents were added was immaterial. After

[1] Details of the technique used and application of the method to a number of rabbit anti-sheep cell sera of varying potency are being gathered into a separate publication.

[2] Kindly carried out by Mrs. Katherine E. Smith of the Hematological Laboratory, Presbyterian Hospital.

[3] S, with appropriate type numeral, indicates type specific polysaccharide of pneumococcus.

TABLE I *a*

Proportions of Hemolysin, Complement, and Red Cells in Hemolysis

Experiment No.	1*	2*	3*
C'1 N† per ml. of guinea pig serum, γ	50	50	50
Fraction of ml. for complete hemolysis of 0.2 ml. sensitized cells	0.004	0.004	0.005
C'1 N necessary‡ for complete hemolysis of sensitized cells, γ	0.20	0.20	0.25
C'1 globulin necessary for hemolysis, γ	1.26	1.26	1.58
Molecules§ of C'1 necessary for hemolysis	5.1×10^{12}	5.1×10^{12}	6.4×10^{12}
Hemolysin N combined per 0.2 ml. of sensitized cell suspension, γ	0.03	0.03	0.03
Hemolysin globulin used for sensitization, γ	0.19	0.19	0.19
Molecules‖ of hemolysin used for sensitization	7.3×10^{11}	7.3×10^{11}	7.3×10^{11}
Number of sheep red cells in 0.2 ml. sensitized suspension	3×10^8	2×10^8	2×10^8
Molecules of C'1 available for hemolysis of single red cell	17,000	26,000	32,000
Molecules of hemolysin combined with single red cell in sensitization and hemolysis	2400	3700	3700

* Details in Paper I (1).

† Complement N, combining component N, "mid-piece" N. The mean extrapolated value, 0.05 mg. per ml. is used (1).

‡ Together with such accessory components as were present in the guinea pig serum dilution actually used.

§ The molecular weight of the combining component of complement is taken as 150,000 (footnote 5).

‖ The molecular weight of antibodies produced in the rabbit is approximately that of the principal component of normal globulin, or 158,000 (5–7).

TABLE I *b*

Continuation of Table I a

Type of ellipsoid and cross section assumed for hemolysin molecule	Cross sectional area of hemolysin molecule	Total cross sectional area of 2400 hemolysin molecules	Red cell surface* occupied by 2400 hemolysin molecules	Total cross sectional area of 3700 hemolysin molecules	Red cell surface occupied by 3700 hemolysin molecules
	cm.$^2 \times 10^{10}$ †	*cm.*$^2 \times 10^{10}$	*per cent*	*cm.*$^2 \times 10^{10}$	*per cent*
Prolate, elliptical cross section	0.0088	21	7.5	32	12
Prolate, circular cross section	0.0009	2.2	0.8	3.3	1.2
Oblate, elliptical cross section	0.0018	4.3	1.5	6.6	2.3
Oblate, circular cross section	0.0210	50	18	77	28

* Average sheep cell radius 2.6×10^{-4} cm. (8); calculated by formula 2, reference 9, in simplified form, area = 5/12 A^2, in which A = 1/2 cell diameter, area of sheep red, cell = 2.8×10^{-8} cm.^{2}

† Calculated with the aid of the following data: asymmetry factor 1.5, partial specific volume 0.745, as for other rabbit antibodies (6, 7); for prolate cases, ratio of axes 1:10, for oblate cases 1:12 (reference 7, Table IV).

TABLE II

Titration of Complement and Hemolysin

Hemolysin	Hemolysin N	Complement, ml.				
		10×10^{-3} C'1 N = 0.5γ	6.25×10^{-3} C'1 N = 0.3γ	4×10^{-3} C'1 N = 0.2γ	2.5×10^{-3} C'1 N = 0.13γ	1.67×10^{-3} C'1 N = 0.08γ
ml. $\times 10^{-5}$	γ					
6	0.03	c	c	c	c	ac
4	0.02	c	c	c	c	m
3	0.015	c	c	c	ac	m
2	0.01	c	c	ac	m	sl
1.5	0.008	c	c	st	sl	tr
1	0.005	st	m	sl	0	0
0.75		m	sl	sl	0	0
0.5		sl	tr	0	0	0
0		0	0			

Symbols: 0 = no hemolysis, tr = trace, sl = slight, m = moderate, st = strong, ac = almost complete, c = complete hemolysis.

TABLE III

Fixation of Complement by S III[3] and Antipneumococcus Type III Rabbit Serum

Antibody N	10×10^{-5} ml. hemolysin, or 0.05γ hemolysin N							
	0.1γ S III				0.01γ S III			
	Complement, ml.				Complement, ml.			
	10×10^{-3} C'1 N = 0.5γ	6.25×10^{-3} C'1 N = 0.3γ	4×10^{-3} C'1 N = 0.2γ	2.5×10^{-3} C'1 N = 0.13γ	10×10^{-3} C'1 N = 0.5γ	6.25×10^{-3} C'1 N = 0.3γ	4×10^{-3} C'1 N = 0.2γ	2.5×10^{-3} C'1 N = 0.13γ
γ								
5	0 0	0 0	0 0	0 0	st c	tr sl	0 0	0 0
3	0 0	0 0	0 0	0 0	m c	0 0	0 0	0 0
2	0 0	0 0	0 0	0 0	sl c	0 0	0 0	0 0
1	0 0	0 0	0 0	0 0	sl c	0 0	0 0	0 0
0.6	0 0	0 0	0 0	0 0	0 c	0 0	0 0	0 0
0.4	0 st	0 0	0 0	0 0	0 ac	0 0	0 0	0 0
0.2	tr c	0 0	0 0	0 0	0 st	0 0	0 0	0 0
0.12	ac c	0 m	0 0	0 0	ac c	0 m	0 0	0 0
0.08	c c	st c	tr m	0 0	c c	m c	0 m	0 0
0.04	c c	c c	m c	0 m	c c	c c	m ac	0 0
0.024	c c	c c	ac c	0 c	c c	c c	m c	0 tr
0.016	c c	c c	c c	0 c	c c	c c	ac c	sl
0	c c	c c	c c	0 st	c c	c c	c c	0 sl
	3×10^{-5} hemolysin, or 0.015γ hemolysin N							
5	0 0	0 0	0 0		c c	tr sl	0 0	
3	0 0	0 0	0 0		st ac	0 tr	0 0	
2	0 0	0 0	0 0		m st	0 0	0 0	
1	0 0	0 0	0 0		m st	0 0	0 0	
0.6	0 0	0 0	0 0		sl m	0 0	0 0	
0.4	0 0	0 0	0 0		0 sl	0 0	0 0	
0.2	m st	0 0	0 0		tr m	0 0	0 0	
0.12	ac c	m ac	tr sl		st c	sl m	0 0	
0.08	c c	c c	sl st		c c	st ac	sl m	
0.04	c c	c c	c c		c c	c c	c c	
0	c c	c c	c c		c c	c c	c c	

Symbols: 0 = no hemolysis, tr = trace, sl = slight, m = moderate, st = strong, ac = almost complete, c = complete hemolysis.

45 minutes at 37°C. each tube received a mixture of 0.1 ml. of 4 per cent sheep red cell suspension and 0.1 ml. containing the volume of hemolysin given in the subheading of Table III. Control rows of tubes containing saline instead of S III were included with each complement dilution. These showed complete hemolysis at the end except for the 2.5×10^{-3} complement dilution, which was unhemolyzed at the first reading and not entirely hemolyzed at the end. This dilution is therefore not used in computing reacting quantities and is included in the table both to show the limit of sensitivity of this mode of titration and to indicate an uncertainty in the calculations. If 0.13 γ of C'1 N did not always suffice for complete hemolysis under the conditions described it is equally uncertain whether all of the C'1 N present in the system was taken up in complete inhibition of hemolysis. At this stage it would appear premature to attempt any correction for these unknown and apparently variable factors, especially as this would involve deductions at each end of the hemolysis scale which might be equal and thus cancel out. Any lack of homogeneity in the complement combining component would also have to be considered in this connection.

In each column of Table III the first row of symbols indicates the reading after all control tubes, except those mentioned above, were completely hemolyzed. The second row of symbols shows the reading after 10 minutes' additional standing at room temperature, after which there was no appreciable change. The first readings appear to give more sensitive indications of zone effects than the final ones and are included for possible future reference.

It is evident from Table III, subject to the uncertainties previously mentioned, that as little as 0.12 γ of anticarbohydrate N in antipneumococcus Type III rabbit serum may take up as much as 0.2 γ of complement N, or complement combining component (C'1) N in the reaction of 0.1 γ of S III with the antibody. Similarly 0.2 γ of anticarbohydrate N and 0.1 γ of S III take up 0.3 γ of C'1 N in combining. At these high dilutions the solutions remain crystal-clear until the hemolytic system is added. With the above proportions of S III and antibody the sensitivity of the test for complete fixation of complement decreases to 0.2 γ of antibody N if the hemolysin in the hemolytic system is reduced from 0.05 γ to 0.015 γ of N. This difference in sensitivity vanishes when only 0.01 γ of S III is used as antigen possibly because the S III-antibody proportions are then more nearly equivalent at this end of the reaction range (10). The limit of sensitivity as regards antibody N remains the same, however, as at the higher level of S III concentration. It is also to be noted that 0.01 γ of S III does not suffice for the complete fixation of as much as 0.5 γ of C'1 N between the limits of antibody concentration used, while a definite zone of complete fixation occurs with 0.3 γ of C'1 N extending over a 10- to 15-fold range of antibody concentration. In the series in which 0.1 γ of S III was used, however, 0.5 γ of C'1 N was readily taken up at antibody N levels of 0.4 γ and above.

In Table IV are given similar data for Ea and anti-Ea rabbit serum. This

portion of the experiment was carried out in the same way as the preceding part. First readings, as recorded in Table III, are omitted since there was little difference except in column 2 of the table. A series of titrations was also carried out with 0.01 γ of Ea N, but only traces of complement were taken up. This is in accord with the far lower antibody N:Ea ratios which obtain in the Ea-anti-Ea precipitin reaction (11) than the ratios characteristic of the S III-anti-S III reaction (10). Nevertheless, in the Ea-anti-Ea reaction, a typical antigen-antibody system, the smallest amount of antibody N detectable with certainty was 0.12 γ, as in the S-anti-S

TABLE IV

Fixation of Complement by Egg Albumin and Anti-Egg Albumin Rabbit Serum

Antibody N	0.1γ egg albumin N						
	10×10^{-5} ml. hemolysin, or 0.05γ hemolysin N				3×10^{-5} ml. hemolysin, or 0.015γ hemolysin N		
	Complement, ml.				Complement, ml.		
	10×10^{-3} C'1 N = 0.5γ	6.25×10^{-3} C'1 N = 0.3γ	4×10^{-3} C'1 N = 0.2γ	2.5×10^{-3} C'1 N = 0.13γ	10×10^{-3} C'1 N = 0.5γ	6.25×10^{-3} C'1 N = 0.3γ	4×10^{-3} C'1 N = 0.2γ
γ							
5	m	0	0	0	0	0	0
3	m	0	0	0	0	0	0
2	0	0	0	0	0	0	0
1	m	0	0	0	0	0	0
0.6	m	0	0	0	0	0	0
0.4	m	0	0	0	0	0	0
0.2	st	0	0	0	0	0	0
0.12	c	sl	0	0	m	0	0
0.08	c	c	sl	0	st	m	m
0.04	c	c	c	sl	c	ac	st
0.024	c	c	c	ac	c	c	ac
0	c	c	c	st	c	c	ac

Symbols as in Tables II and III.

system, and again the antigen-antibody complex was found capable of taking up at least an equal weight of complement N.

Additional Data on Complement Fixation.—In a number of earlier experiments the quantity of complement was held constant at 0.25 ml. of a 1:10 dilution, or approximately 1.3 γ of C'1 N, while 3 to 10 minimal hemolytic doses of hemolysin were used. Under these conditions the maximum sensitivity for anti-S I was reached at 1.8 γ of antibody N with 0.25 γ of S I, but if as little as 0.025 or 0.016 γ of S I was used the minimum amount of antibody N which completely fixed 1.3 γ of C'1 N was 3 γ. Later experiments with 0.5 γ of C'1 N and 0.1 γ of S I showed a lower limit of 0.2, 0.4, and 0.4 γ of antibody N with three different antisera, while with 0.01 γ

of S I the values were 0.12 or less, 0.8, and 0.8 γ. With S II and an antipneumococcus Type II rabbit serum the lowest limits of complete fixation of approximately 0.5 γ of C'1 N were 1.5, 0.5, and 0.3 γ of antibody N with 1, 0.1, and 0.01 γ of S II respectively. Repetition of the experiment with another serum gave 1.1, 0.3, and 0.3 γ of antibody N.

A sample of purified Type I pneumococcus rabbit anticarbohydrate, 193B, was prepared by dissociation with barium hydroxide (12) after previous extraction of a portion of the washed precipitate with 15 per cent salt solution. The solution contained 0.44 mg. of N per ml., of which 0.4 mg. was antibody N. A dilution containing 10 γ of antibody N fixed 1.3 γ of C'1 N completely with 2.5 γ of S I as antigen. The lower limit of sensitivity for antibody was not reached, as smaller amounts of S I were not tested. With the same antibody solution, 20 γ of antibody N fixed 1.3 γ of complement N completely with S I quantities ranging from 20 γ down to 0.03 γ. Similar proportions and limits were found in the S III-anti-S III system, in which 15 γ of antibody N (diluted antiserum) fixed 1.3 γ of C'1 N with quantities of S III ranging from 25 γ down to 0.025 γ. As nearly as can be deduced from their studies with larger "units," Goodner and Horsfall (13) found combining proportions of similar range and order of magnitude with S I and antipneumococcus Type I rabbit serum of known antibody content.

With the corresponding homologous type specific polysaccharides two antipneumococcus Type III rabbit sera and one of two Type VIII sera gave fixation of 1.3 γ of C'1 N in and below the zone of visible precipitation, but cross reactions between S III and anti-S VIII or S VIII and anti-S III could not be demonstrated either by precipitation or by complement fixation. On the other hand S II precipitated and fixed complement with rabbit antiserum to Friedländer's bacillus Type B over a narrower range than the Friedländer B polysaccharide, but the latter substance failed to precipitate or fix complement with the rabbit anti-Pn II serum used although this contained 1.74 mg. of anti-S II per ml. (*cf.*, also 14).

Highly purified bovine antipneumococcus Types I and II antibody solutions (12) also showed relations between antigen, antibody, and complement similar to those exhibited by antibody formed in the rabbit. With 0.04 γ of S I, bovine anti-S I fixed 1.3 γ of C'1 N down to 3.6 γ of antibody N, while bovine anti-S II showed a similar lower limit with 0.02 γ of S II. This result indicates that the high molecular weight of horse antipneumococcus antibodies (5, 6) is not responsible for the failure of these antibodies to fix complement, since the bovine antipneumococcus antibody has the same high molecular weight (6).

DISCUSSION

It was shown in the preceding paper that the combining component of guinea pig complement could be measured in weight units and that more than 40 per cent of complement, by weight, could be added to antigen-antibody precipitates with maintenance of volumes of the reacting components at levels convenient for precise analytical measurement. However, it was scarcely feasible to use larger and larger volumes of guinea pig serum in order to determine the maximum amount of complement capable of reaction with known quantities of antigen and antibody. Instead, the usual technique of the complement fixation test, with its great delicacy and reproducibility under strictly controlled conditions, appeared capable of affording the necessary information, and could, by virtue of this very delicacy, be used to determine at the same time the lower limits of reactivity and the combining proportions of the reacting components. By the use of antigen and antibody solutions of known content and with the aid of the values for complement obtained in the preceding paper the actual quantities of antigen, antibody, and complement could be calculated in weight units for each significant dilution and mixture. In several experiments the number of red cells in the hemolytic system was counted and the amount of hemolysin estimated.[1]

Since the hemolysis of sensitized red cells by complement depends upon other factors in guinea pig serum as well as the combining component of complement (2, 3, 4, 15) it is impossible to define the mechanism of hemolysis solely on the basis of the number of red cells and the actual quantities of hemolysin and complement combining component. The cell count is easily made, however, and the other two variables may now be estimated with a fair degree of certainty. Their interrelationships, under the conditions used, are set forth in Table I. Additional data on the relative amounts of hemolysin and complement combining component required as the test of complete hemolysis approaches its limit of delicacy, will be found in the first portion of Experiment 10. It is evident that at extreme dilutions the actual amounts of hemolysin and complement combining component approach equality, but that as the test is ordinarily carried out far more combining component than hemolysin is present. The latter may be considered as actually combined with the red cells in the experiments summarized in Table I as only twice the minimum sensitizing quantity was used, but it seems probable that, except at the highest dilutions of guinea pig serum shown in Table II, complement was present in excess. Possibly only an amount equal to the hemolysin, or a small multiple of this amount, entered into actual combination with the sensitized cells (*cf.* 16). For this

reason, if any portion of the complement functions enzymatically in hemolysis, it is probably not the combining component, for at least as much of this protein as hemolysin must be present. This does not resemble catalysis. Moreover, the combining component of complement actually unites in stable union with sensitized red cells, as with other antigen-antibody systems (17).[4]

The available data regarding the antibody molecule (6, 7) are such that an uncertainty remains regarding its shape. The experimental value for the frictional coefficient or asymmetry factor, however, permits the calculation of molecular areas on the assumption of either oblate or prolate spheroidal shape. The area occupied by such molecules on the red cell surface would depend upon their attachment in either the endwise or lateral position. In Table 1 *b* calculations of the occupied surface are therefore given for each position of the oblate or prolate spheroidal molecule. Intermediate positions are also conceivable. Depending, then, on which calculation is used, the experimental data indicate that roughly from 0.8 to 28 per cent of the sheep cell surface is occupied by hemolysin under the conditions employed. Since two "units" of hemolysin were actually used, complete hemolysis is possible with 0.4 to 14 per cent of the cell surface combined with antibody. While these figures are not to be taken too literally they do at least indicate by roughly a power of ten that sensitization need not involve a coating of the entire red cell surface. The findings are therefore in accord with the "key spot" theory of Abramson (18).

Brunius (19) has calculated the number of the Forssman antibody molecules required to sensitize a single red cell for hemolysis, finding the number as only 30 and the fraction of red cell surface covered as 0.001 per cent. The area calculations were made on a different basis, but the number of molecules of hemolysin was calculated from a single preparation which showed about ten times the activity per γ of N as four other lots isolated by Brunius, the best preparation of Locke and Hirsch (20), and our own sera, judged by their antibody content. When it is considered that the 3000 molecules of hemolysin indicated in Table I represent double the number necessary for complete hemolysis, and that Brunius used 90 per cent hemolysis as the end-point, the agreement among the less active preparations is moderately close. However, sheep red cell stromata may contain a number of antigens. While the antibodies resulting from the injection of these antigens into rabbits are capable of combining with stromata[1] it does not follow that all of the antibodies actually prepare the cell for hemolysis. The one result used by Brunius for his calculations may there-

[4] C'1 N is taken up by sheep cell stromata in their reaction with hemolysin (unpublished experiment).

fore be the more significant for the process of hemolysis itself, while the larger number of molecules found in the present experiments represents combination between the red cell and total antibody under the conditions of the hemolytic test as actually carried out with rabbit hemolysin.

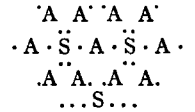
Although the present experiments have only limited application in the study of immune hemolysis they afford a much more complete picture of the fixation of complement in certain immune reactions. With the use of 50 γ as the average complement combining component nitrogen content of guinea pig serum (1) it is possible to interpret complement fixation tests carried out on a number of sera of known antibody content (for example, Experiment 10). From the limiting dilutions of complement, antigen, and antibody at which the quantity of complement used in each instance was completely taken up it would seem evident that at least as much complement nitrogen as antibody nitrogen may enter into the reaction. Experiments over a wide range with S I and anti-S I also showed that quantities of complement larger than at the limiting dilution could be fully taken up only in the presence of larger amounts of antibody, and that the antigen could be varied relatively independently above a minimum value. It is also clear that the final colloidal state of the system failed to influence the proportions in which complement combined, since the quantities of complement N added to antigen-antibody mixtures yielding specific precipitates with 0.4 to 0.6 mg. of N were of the same relative magnitude as in the clear solutions containing only fractions of a γ of reactants. A chemical, rather than physical, explanation for the fixation of complement is therefore indicated and Ehrlich's concept of complement activity is confirmed in this respect both qualitatively and quantitatively.

At the time Ehrlich's theories were proposed the nature of antigens and antibodies was uncertain and methods of measurement were purely relative. After the introduction of quantitative, absolute methods (21) a large body of precise information regarding immune reactions was assembled, and with the recognition of the protein nature of antibodies it became possible to formulate chemical theories of antigen-antibody reactions which were in accord with modern concepts of the structures of the reacting substances (21, 22). Now that the combining component of complement has been added to the list of immune substances measurable in weight units it is possible to put these theories to the severe test of their adaptability to the inclusion of complement, hitherto neglected for the sake of simplicity.

It was shown by the work of this laboratory that the precipitin (23) and agglutinin (24) reactions might be quantitatively expressed by equations derived from the mass law. Chief among the assumptions made was that

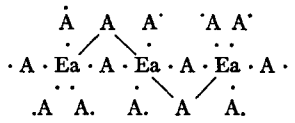
both antigen and antibody were multivalent with respect to each other; that is, that each possessed two or more groupings reactive with the other. After the molecular weights of antibodies became known (5, 6) it was possible to assign empirical formulas to specific precipitates formed at certain reference points in the precipitin reaction range (25). Now that both the molecular weight[5] and the reacting quantities of the combining component of complement are known it is possible to fit this component into the above quantitative theory with little difficulty.

In a number of papers (23, 11, 21 *b*, and others) two-dimensional representations of three-dimensional aggregates formed by the union of multivalent antigen with multivalent antibody were depicted somewhat as follows:

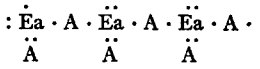


[1]

in which S represents specific polysaccharide or antigen and A represents antibody, or the compounds Ea_nA_{4n+2} and $(EaA_2)_n$ in the antibody excess and equivalence zones respectively (21 *b*):



[2]



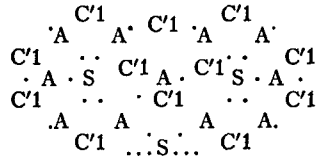
[3]

In the last two formulas the valence of Ea is taken as 6 and that of A limited to 2 for simplicity. A somewhat similar scheme has been suggested by Marrack (22) and more recently an analogous one by Pauling (26), in which the valence of antibody was limited to two.

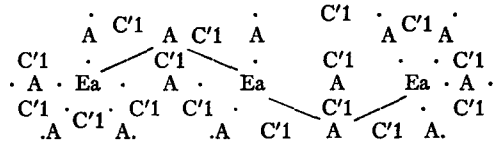
The assumption is now made that the combining component of complement, whether it possesses a single reactive grouping, or whether like antigen and antibody it carries two or more reactive groupings, is capable only of loose, easily dissociable combination with dissolved antibody. This is in accord with numerous observations that complement is not fixed by antigen or antibody alone (2, 3). How then, is it possible to account for the firm fixation of amounts of complement equimolecular[5] with those of antibody by antigen-antibody combinations whether or not these actually separate from solution?

[5] Private communication from Professor E. E. Ecker and Dr. L. Pillemer (*cf* Table I *a*).

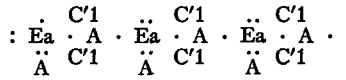
It will be recalled that in the development of the quantitative theory of the precipitin reaction (21, 23) it was assumed that multivalent antigen combined with multivalent antibody in a series of competing bimolecular reactions to build up large aggregates like those illustrated, and that the process might be well advanced before the aggregates separated from solution. At high dilutions, particularly with rabbit antisera, which form specific precipitates of appreciable solubility (10, 11), the aggregates would not necessarily separate. Whether or not precipitation occurs, the formation of such multimolecular aggregates would bring together and hold myriads of antibody molecules. But by this act any molecules of complement combining components (C′1) present would be surrounded by antibody molecules. In this way a linkage between C′1 and A, ordinarily dissociated at once when taking place between single molecules, might become stabilized when occurring between C′1 and *n*A. Stabilization might result either through the attraction of approaching ionized groupings of opposite sign, through hydrogen bonding, through spatial accommodation of large groupings on C′1 and A, or through the presence, on C′1, as on antigen and antibody, of more than one grouping capable of reacting with A molecules brought into apposition. The result might then be represented in the two-dimensional schemes, 1 *a*, 2 *a*, and 3 *a*, corresponding to 1, 2, and 3 above.



[1 *a*]



[2 *a*]



[3 *a*]

An analogous instance of loose, dissociable combination, capable of conversion into firm union by addition to multivalent antigen-antibody aggregates, is found in the apparently "univalent" antibody known to

occur in many immune sera (11, 23, 27, and other papers). This residual antibody, remaining after fractional absorption of many sera with antigen, forms only easily dissociated, soluble compounds with antigen. However, when multivalent, or complete, antibody is present, the "univalent" or residual antibody may add to the resulting antigen-antibody aggregates in firm, relatively undissociated union and be precipitated just as is the multivalent antibody.

In the above discussion and in the graphic representations complement has been considered, for simplicity's sake, solely in terms of its relation to antibody. This suffices to explain its behavior provided the quantity of C'1 combined does not exceed that of A. However, the data indicate that a somewhat larger proportion of C'1 may be added when relatively more complement is present. It is therefore probable that, at least under such conditions, antigen molecules may participate in binding C'1 firmly in the antigen-antibody aggregate, and it is not excluded that antigen plays some part under all circumstances. It is known, however, (25) that throughout practically the entire range of the precipitin reaction more antibody molecules than antigen molecules are present, the proportion ranging from two- to three-fold in the equivalence zone to 6 times or greater in the region of large antibody excess. It would therefore seem reasonable to ascribe to the network of antibody molecules the major burden in the firm linkage of complement to antigen-antibody aggregates, the more so as the complement uptake is influenced to a far greater extent by the quantity of antibody present than by the amount of antigen.

Other evidence in accord with the conception of complement fixation as a consequence of aggregate formation by combination of multivalent antigen with multivalent antibody is furnished by the behavior of the pneumococcus Type III and Type VIII polysaccharide-rabbit antisera reactions, and of the Type II and Friedländer Type B polysaccharide reactions in rabbit antisera. In each homologous reaction with the appropriate specific polysaccharide complement was fixed over a wide range (see page 701), extending in the Type II and Type VIII sera to high dilutions at which visible precipitation no longer occurred. However, no visible cross precipitation occurred, even at relatively low dilutions, between S III and anti-Type VIII sera, or between S VIII and anti-Type III sera, nor was complement fixed in these cross tests. On the other hand S II gave specific precipitation and complement fixation with a Friedländer B antiserum, but the Friedländer B polysaccharide neither precipitated nor fixed complement with Type II antipneumococcus rabbit serum. At least in these instances the possibility of multivalent antigen-antibody aggregate formation seems to

be a prerequisite for complement fixation even at high dilutions at which the aggregates fail to separate.

It is, however, well known that complement is not fixed by all antigen-antibody reactions which may be expressed in terms of aggregate formation by union of multivalent antigen with multivalent antibody. Although some antigen-horse antibody systems fix complement, pneumococcus specific polysaccharides react with pneumococcus anticarbohydrate from the horse and with rabbit anticarbohydrate damaged by acid (28) to form aggregates which do not bind complement. The same polysaccharides react with unaltered rabbit and bovine anticarbohydrate with fixation of large amounts of C′1. Since almost all antigen-rabbit antibody systems fix C′1 it would seem possible that rabbit antibody best fulfills the steric requirements for the firm union of C′1 within the molecular network of the antigen-antibody aggregates. This cannot be entirely due to the equimolecular size of C′1 and rabbit antibody, since bovine A, which also permits complement fixation, is known to have a much greater size. Possibly accompanying serum lipids play a part, as postulated by Goodner and Horsfall (13).

According to these views, then, complement combining components would differ from normal globulin in the possession of one or more groupings capable of forming loose dissociable unions with individual antibody (and perhaps antigen) molecules, but yielding firm, difficulty dissociable combinations, with the exceptions noted, when surrounded by antibody (and perhaps antigen) molecules. In this way the failure of C′1 to be taken up appreciably by antigen or antibody alone is readily accounted for, also the fixation of C′1 in qualitatively and quantitatively similar fashion by all but a relatively limited number of antigen-antibody combinations. It is even conceivable that C′1 would unite with equal firmness with normal globulin were there a means of bringing a sufficient number of such molecules into suitable apposition and holding them there.

The phenomenon of complement fixation may thus be fitted into the framework of the quantitative precipitin (and agglutinin) theory with little stretching of postulates which had already shown themselves of some utility. While this affords no proof of the theory it at least justifies its use as a guide for further experiments in a field which the more conventional and alternative theories have failed to clarify.

SUMMARY

1. The molecular quantities of hemolysin and complement combining component or components (C′1) involved in hemolysis have been calculated on the basis of new, quantitative, absolute methods of analysis.

2. Molecular combining ratios between antigen, antibody, and C'1 have been established.

3. The data are shown to be in accord with the theory of combination of multivalent antigen with multivalent antibody.

4. The fixation of complement by antigen-antibody combination is qualitatively and quantitatively accounted for on this basis.

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