QUANTITATIVE CHEMICAL STUDIES ON COMPLEMENT OR ALEXIN

III. Uptake of Complement Nitrogen under Varying Experimental Conditions*

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In the first paper of this series (1) it was shown that the combining component or components of guinea pig complement could be estimated in weight units by determination of the quantity of nitrogen added to certain specific precipitates formed in the presence of sufficient active complement. In the second paper (2) the data so obtained were used to trace the relationships of the combining component (C'1) of complement to red cell and hemolysin in hemolysis. It was also shown, by tests at high dilutions with known quantities of antigen, antibody, and complement, that fixation of C'1 in antigen-antibody combination could occur in amounts equimolecular with the antibody, or even greater, and that the fixation was relatively little influenced by quantities of antigen above a necessary minimum. An explanation of complement fixation was also given in terms of the union of multivalent antigen with multivalent antibody, the same concept which has contributed to the understanding of the precipitin and agglutinin reactions (3–5) and their practical application.

The values given (1) for the average content of C'1 N in guinea pig serum, 0.04 to 0.06 mg. of N per ml., were based upon the addition of C'1 N to antigen-antibody precipitates formed in the guinea pig serum, with the combining proportions so chosen that an excess of antibody remained unprecipitated. While the specific precipitates were obtained in finely divided form in this way and could be the more easily washed, the presence of excess antibody introduced an uncertainty, since it was possible that complement (C') permitted a given

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amount of antigen to combine with more antibody than in the absence of C′, with a resulting increase in nitrogen due to antibody uptake rather than C′1 uptake. It was stated in (1), page 690, that this uptake had no basis, and the substantiating experiments are given in the present report. These provided data on the fixation of C′1 N by precipitates formed in guinea pig serum in the region of antigen excess and on the uptake of C′1 N by preformed washed specific precipitates. Experiments are also described on the effect of varying reaction periods and on the relation of the amount of C′1 N bound to the quantity of precipitate used for fixation.

Materials and Methods

Guinea pig serum was used as complement (C′). Inactivated complement (iC′) was prepared by heating the serum at 56°C. for 50 minutes (thermometer in the serum). Shorter inactivation was effective in abolishing the hemolytic activity of C′ but did not always reduce the amount of combining component (C′1) to a minimum (1). C′ and iC′ were allowed to stand overnight and were centrifuged again in the cold[1] before using.

Anti-egg albumin and antipneumococcus Type III rabbit sera were used with egg albumin (Ea) and the specific polysaccharide of Type III pneumococcus (S III), respectively, as immune systems. The rabbit antisera were not inactivated, as they were employed at dilutions at which their C′1 N content could not be measured.

Quantitative precipitin estimations were carried out as described in numerous papers (for example, 1, 6) with accurately measured quantities of serum and antigen. Analyses were usually run in triplicate, and the specific precipitates were washed three times instead of twice, as was also done in (1). Comparisons were made of the amount of specific N precipitated in the presence of known volumes of C, iC′, and saline. The difference between the values obtained with C′ and iC′ was considered due to complement combining component nitrogen (C′1 N).

In all except the experiments with preformed specific precipitates antiserum was thoroughly mixed with C′ (or iC′) and saline before addition of antigen. Hemolytic "units" were measured as in (1).[2]

EXPERIMENTAL

Experiment 11. Comparison of Reaction Periods of 1, 2, and 3 Hours.—Temperature, 19.5°C. Complement "titer," 250 "units." Portion of C′ inactivated for 50 minutes at 56°C. (iC′). Antipneumococcus Type III rabbit serum B 6, diluted with 5.5 volumes of saline, pooled with corresponding dilution of serum 6.06_{2b}. S III dilution contained 0.04 mg. per ml. All determinations in triplicate except blanks. Tube contents were mixed frequently during period of standing.

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[1] In an International Equipment Company refrigerated centrifuge.

[2] The sheep red cells used were kindly furnished by Miss Edna Baker of the Wassermann Laboratory, Presbyterian Hospital.

Hrs. at 19.5°C.	1								2		3	
C′, *ml.*	3.0	3.0					3.0	5.0	3.0	5.0	3.0	5.0
iC′, *ml.*			3.0	3.0		3.0						
Serum dilution, *ml.*	1.0			1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
S III dilution, *ml.*		1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Saline, *ml.*	3	3	3	3	5	2	2		2		2	
N precipitated, *mg.*	0.006	0.004	0.012	0.008	0.456 0.500 0.476	0.518 0.508 0.514	0.588 0.588 0.586	0.630 0.622 0.630	0.590 0.586 0.578	0.626 0.638 0.622	0.582 0.576 0.592	0.622 0.618 0.620
Mean	0.005		0.010			0.513	0.587	0.627	0.585	0.629	0.583	0.620
Subtraction of blank						0.010	0.005	0.008*	0.005	0.008*	0.005	0.008*
Specific N precipitated, *mg.*						0.503	0.582	0.619	0.580	0.621	0.578	0.612
Subtraction of iC′ value							0.503	0.510†	0.503	0.510†	0.503	0.510†
C′1 N precipitated, *mg.*							0.079	0.109	0.077	0.111	0.075	0.102
C′1 N per ml. guinea pig serum taken, *mg.*							0.026	0.022	0.026	0.022	0.025	0.020

Hemolytic "units" left in C′ supernatants, $<<15$.

* $0.005 \times 5/3$.

† In this experiment the saline control values varied unaccountably. It was therefore assumed that the iC′ N value, 0.503, was 0.01 mg. greater than the saline series should have been, as was frequently found in (1) for 3 ml. of iC′. For 5 ml., 2/3 of 0.01 was added to the iC′ series value for 3 ml. (column 7).

Experiment 12. Fixation of Complement by Specific Precipitate Containing Excess Antigen.—Temperature, 22°C. Complement "titer," 285 "units" per ml. Anti-Ea pool C rabbit serum used, diluted with 2 volumes of 0.9 per cent saline. Ea solution in saline contained 0.058 mg. Ea N per ml. The supernatant from a mixture of 1.0 ml. each of Ea and anti-Ea contained a trace of Ea as indicated by a positive test with antiserum and a negative test with Ea. In the experiment itself the contents of the tubes were thoroughly mixed and Ea was added last.

No. of tubes	1	1	1	1	1	2	2	3
C′, *ml.*	3.0	3.0						3.0
iC′, *ml.*			3.0	3.0			3.0	
Serum dilution, *ml.*	1.0		1.0		1.0	1.0	1.0	1.0
Ea dilution, *ml.*		1.0		1.0		1.0	1.0	1.0
Saline, *ml.*	3	3	3	3	6	5	2	2
N precipitated, *mg.*	0.010	0.008	0.016	0.018	0.002	0.524 0.510	0.526 0.522	0.620 0.620 0.612
Mean	0.009		0.017		0.002	0.517	0.524	0.617
Subtraction of blank						0.002	0.017	0.009
Specific N precipitated, *mg.*						0.515	0.507	0.608
Subtraction of iC′ series value								0.507
C′1 N precipitated, *mg.*								0.10

Hemolytic units left in C′ series supernatants, $<<25$.

Experiment 13.—Since this experiment was designed to supply comparative data on C'1 N estimations in active guinea pig serum with antigen-antibody mixtures in different proportions and with preformed precipitates, no analyses were run in inactivated complement. C'1 N was calculated by addition of 0.01 mg. of N per 3 ml. of C' to the saline control values (*cf.* 1). Temperature, 22°C. Complement "titer," 285 "units" per ml. Rabbit antipneumococcus Type III serum B 6 used, diluted with 3.5 volumes of saline. The S III solutions contained 0.04 and 0.12 mg. per ml. Reaction time, 1 hour and 20 minutes, with frequent mixing.

Column	1	2	3	4	5	6	7	8	9	10	11	12
No. of tubes	1	1	1	3	3	3	3	3	3	3	3*	3*
C', *ml.*		5.0	5.0		3.0	5.0		3.0	5.0	3.0	5.0	
Serum dilution, *ml.*	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
S III, 0.04 mg. per ml., *ml.*			1.0	1.0	1.0	1.0					1.0	1.0
S III, 0.12 mg. per ml., *ml.*								1.0	1.0	1.0		
Saline, *ml.*	7	2	2	6	3	1	6	3	1	5	3	
N precipitated, *mg.*	0.008	0.016	0.014	{0.490 0.472 0.496	{0.584 0.582 0.578	{0.626 0.626 0.622	{0.692 0.694 0.698	{0.794 0.790 0.794	{0.860 0.856 0.842	{0.570 0.578 0.574	{0.600 0.604 0.616	
Mean	0.008	0.015		0.486	0.581	0.625	0.695	0.793	0.853	0.574	0.607	
Subtraction of blank				0.008	0.009†	0.015	0.008	0.009†	0.015	0.009†	0.015	
Specific N precipitated, *mg.*				0.478	0.572	0.610	0.687	0.784	0.838	0.565	0.592	
Subtraction of calculated iC' value					0.488	0.495		0.697	0.704	0.488	0.495	
C'1 N precipitated, *mg.*					0.08	0.12		0.09	0.13	0.08	0.10	

Hemolytic units left in entire supernatants of first four C' series: $<<16$ in each; in supernatant from precipitate series with 3 ml. C' (column 11), $<<16$; in supernatant from precipitate series with 5 ml. C' (last column), <30.

* In these two sets of tubes S III and antiserum were first mixed, allowed to stand 0.5 hour at room temperature, centrifuged in the cold, washed with 3 ml. of chilled saline, and again centrifuged. The washed precipitates were resuspended in the volumes of saline indicated and the guinea pig serum was then added.

† 3/5 × 0.015 (column 3).

Duplicate aliquot portions of 8.0 ml. of the combined supernatants of the tubes in columns 5, 6, and 7 of the protocol, which originally contained an excess of antibody, were mixed with an additional 0.04 mg. of S III and allowed to stand in the cold overnight. The resulting precipitates were centrifuged in the cold and analyzed for N in the usual way. The quantities found were the same, whether or not complement had originally been present: 0.186 mg. in the salt supernatants, 0.194 in the 3 ml. C' supernatants, and 0.192 in the 5 ml. C' supernatants. Excess S III was present in the supernatants from these analyses, indicating that antibody precipitation was complete. The quantity of antibody N present, calculated from the difference between the specific N values in columns 8 and 5, was 0.21 mg.

Experiment 14. Fixation of Complement by Varying Amounts of Specific Precipitate.—Temperature, 25°C. Complement "titer," 250 "units." 75 ml. inactivated at 56°C. (thermometer in serum) for 50 minutes. A specific precipitate was prepared as follows on the day before use: 10 ml. of antipneumococcus Type III rabbit serum B 53, containing 3.56 mg. of antibody N per ml., were diluted to about 35 ml. and mixed with a solution of 2 mg. of S III, an amount chosen to leave antibody in excess.

After 0.5 hour at 0°C. the mixture was centrifuged and the precipitate was washed twice with 35 to 40 ml. of chilled saline, resuspended in 35 ml. of saline containing 1:10,000 merthiolate,[3] and filtered through a loose cotton plug to remove any large lumps. The N content of the suspension (lot A) was 0.54 mg. per ml. Suspensions B and C were prepared from lot A by dilution of 7.5 ml. to 10 and 15 ml., respectively, with saline, while suspension D was prepared by dilution of 2.5 ml. of lot A to 10 ml., and suspension E by dilution of 2 ml. of lot A to 15 ml. As shown in the protocol, the suspensions were mixed with 7 ml. of saline, 7.0 ml. of iC′, and 7.0 ml. of C′ and centrifuged after 2 hours at 25°, with frequent mixing. Triplicate determinations were run in every instance except with the C′ and iC′-saline blanks, on which single estimations were made.

Suspension, 1.5 ml.	A	B	C	D	E
Saline, *ml.*	7	7	7	7	7
N precipitated, *mg.*	0.720 0.708 0.722	0.540 0.542 0.538	0.366 0.360 0.354	0.178 0.180 0.180	0.104 0.092 0.090
Mean	0.717	0.540	0.360	0.179	0.095

Suspension, 1.5 ml.			A	C	E	A	B	C	D	E
iC′, *ml.*	7.0		7.0	7.0	7.0					
C′, *ml.*		7.0				7.0	7.0	7.0	7.0	7.0
Saline, *ml.*	1.5	1.5								
N precipitated, *mg.*	0.008	0.006	0.764 0.758 0.768	0.382 0.376 0.376	0.108 0.104 0.102	0.874 0.868 0.868	0.660 0.666 0.682	0.464 0.478 0.480	0.274 0.276 0.268	0.176 0.178 0.176
Mean			0.763	0.378	0.105	0.870	0.669	0.474	0.273	0.177
Subtraction of blank			0.008	0.008	0.008	0.006	0.006	0.006	0.006	0.006
Specific N precipitated, *mg.*			0.755	0.370	0.097	0.864	0.663	0.468	0.267	0.171
Subtraction of corresponding iC′ series value						0.755	0.560*	0.370	0.185†	0.097
C′1 N taken up, *mg.*						0.11	0.10	0.10	0.08	0.07
Hemolytic "units" remaining in supernatant (of 1750 originally)						100	200	280	430	570

* Rough extrapolation between the A and C series values made by addition of 0.02 mg. N to the corresponding value in salt series (column 3, first part of protocol).

† Rough extrapolation between the C and E series values made by addition of 0.006 mg. N to corresponding value in salt series (column 5, first part of protocol).

Experiment 15. Fixation of Complement by Quantities of S III and Anti-S III in Excess.—Temperature, about 20°C. Complement "titer," 250 "units." 70 ml. inactivated as before. Dilutions of rabbit serum pool B 6, 56 (3.0 mg. of anti-S III N per ml.) were made with saline as follows: lot A, 7.5 ml. serum diluted to 20 ml.; lot B

[3] Manufactured by Eli Lilly and Company, Indianapolis, Indiana.

5 ml. serum to 20 ml.; lot C, 2.5 ml. serum to 20 ml. Corresponding dilutions of S III, were also made up to contain: A, 0.071 mg. S III per ml.; B, 0.048 mg. per ml.; and C, 0.024 mg. per ml. Blanks, 1 tube each:

Serum and S III dilution used	A					B	C				
C′, *ml.*		5.0	5.0					5.0	5.0		
iC′, *ml.*				5.0	5.0					5.0	5.0
Saline, *ml.*	6.5	1.5	1.5	1.5	1.5	6.5	6.5	1.5	1.5	1.5	1.5
Serum dilution, *ml.*	1.5	1.5			1.5	1.5	1.5	1.5			1.5
S III dilution, *ml.*			1.5	1.5					1.5	1.5	
N precipitated, *mg.*	0.002	0.018	0.010	0.004	0.014	0.002	0	0.002	0.010	0.016	0.010
Mean blank N, *mg.*		0.014		0.009				0.006		0.013	

Analyses, in triplicate, with 1.5 ml. serum dilution and 1.5 ml. S III dilution added to each tube:

Serum and S III dilution used	A			B			C		
C′, *ml.*			5.0			5.0			5.0
iC′, *ml.*		5.0			5.0			5.0	
Saline, *ml.*	5			5			5		
N precipitated, *mg.*	1.134	1.166	1.264	0.756	0.762	0.874	0.374	0.382	0.482
	1.138	1.150	1.270	0.768	0.766	0.878	0.360	0.376	0.476
	1.154	1.170	1.264	0.750	0.770	0.882	0.376	0.382	0.492
Mean	1.142	1.162	1.266	0.758	0.766	0.878	0.370	0.380	0.483
Subtraction of blank	0.002	0.009	0.014	0.002	0.011*	0.010†	0	0.013	0.006
Specific N precipitated, *mg.*	1.140	1.153	1.252	0.756	0.755	0.868	0.370	0.367	0.477
Subtraction of iC′ values			1.153			0.755			0.367
C′1 N precipitated, *mg.*			0.10			0.11			0.11

Hemolytic units left in A, B, C complement supernatants, $<<16$.

* Calculated by interpolation from the A and C series blanks.

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DISCUSSION

The data given in Experiment 11 show that uptake of complement combining component nitrogen (C′1 N) by a specific precipitate was complete in 1 hour at room temperature, and that identical quantities of C′1 N were also added when exposure to C′ was continued 1 or 2 hours longer. The apparent solubility effect, previously noted (1), in which less C′1 N per ml. was removed from relatively larger quantities of guinea pig serum, was also found to be

uninfluenced by the period of contact of the reactants within the limits given, since in all cases 0.025 to 0.026 mg. of C'1 N per ml. was taken up from 3 ml. portions of C' and 0.020 to 0.022 mg. per ml. from 5 ml. portions.

It is shown in Experiment 12 that specific precipitates formed in active guinea pig serum by addition of a slight excess of egg albumin to rabbit anti-egg albumin serum took up more nitrogen than was added in the same quantity of inactivated guinea pig serum. The difference, 0.10 mg., for 3 ml. of complement, was of the same magnitude as in previous instances in which an excess of antibody, rather than an excess of antigen, was used (1) (also Experiment 11).

It is also evident from Experiment 13 that identical quantities of C'1 N were removed from active guinea pig serum by sufficient S III-rabbit anti-S III precipitate whether the specific precipitate was formed in the region of excess antibody or in the region of excess S III. The last portion of the experiment also shows that the supernatants containing excess antibody held the same quantity of antibody when saline, inactivated complement, or active complement was present in the original precipitations, showing directly that the combining proportions of S III and antibody are uninfluenced by active complement. In the measurement of C'1 N, therefore, it is immaterial whether a moderate excess of antigen or antibody be used provided the texture of the specific precipitate remains such that efficient washing is possible. In none of the numerous experiments so far recorded, therefore, was the nitrogen uptake in the presence of active complement due to excess antibody and this strengthens the conclusion that the increase observed was actually due to complement combining component.

The data in the last two columns of the protocol of Experiment 13 show that a specific precipitate, freshly formed in the region of antibody excess, was almost as efficient in the fixation of C'1 N as were the corresponding quantities of S III and antibody when added separately. The washed specific precipitate removed the same quantity of C'1 N from the 3 ml. portions of C' as did S III and anti-S III in solution, whereas 0.48 mg. of precipitate N was not sufficient to take out all of the C'1 from 5 ml. of C'. This is shown not only by the C'1 N values in the protocol, but also by the tests for the number of hemolytic units of C' remaining in the supernatants.

It will be remembered that Goodner and Horsfall (7) showed that precipitation and centrifugation greatly impaired the capacity of specific precipitates to fix complement. The results now reported, which show that under suitable conditions specific precipitates may fix as much C'1 N as antigen and antibody added separately, are not at all in conflict with this conclusion. The specific precipitates used in the present studies were prepared in the region of excess antibody so that they might be resuspended in finely divided form after repeated centrifugation, and pains were taken, by frequent mixing of the tube

contents, to ensure prolonged contact of the precipitates with the C′. The observations of Goodner and Horsfall (7) clearly demonstrated the decisive effect of variation of experimental conditions on the fixation of complement, so that the recording of opposite conclusions under widely different conditions is not only to be expected but may be easily accounted for.

In view of the efficient fixation of C′1 by finely divided specific precipitates a study was made in Experiment 14 of the uptake of C′1 N by varying quantities of preformed S III-anti-S III. The relative amounts of C′ and specific precipitate were so chosen that excess C′ would remain. While it is hoped that additional experiments along these lines will aid in a more complete understanding of the mechanism of complement fixation, these initial data seem significant in their relation to other observations (1, 2). It will be noted that in series E 0.097 mg. of specific N fixed 0.07 mg. of C′1 N, or 72 per cent of its own weight of complement combining component, a result approaching in magnitude the uptake of even more than an equal weight of C′1 N under conditions of greatest sensitivity (2). Double the quantity of specific N removed only a little more C′1 N (0.08 mg.), but it is evident from the remaining data that still larger amounts of specific N removed more C′1 N and also a larger number of units of hemolytic activity. More information will be necessary, however, for the evaluation of the relationship between these two measures of complement activity.

In Experiment 15 identical quantities of C′1 N, within the error of the determinations, were removed from 5 ml. of another pool of guinea pig serum by 0.37, 0.76, and 1.14 mg. of specific N, showing that when sufficient precipitate is present to exhaust the C′1 the amount of C′1 N found is independent of the specific N taken.

The several tests described above serve, then, to confirm the validity of the method for the estimation of complement combining component(s) in weight units, and to extend the experimental basis for its use.

Theoretical implications of the large uptake of C′1 N by preformed specific precipitates will be discussed when more data are available.

SUMMARY

1. Quantitative data are given on the effect of variations in the time of contact and the proportions of the reactants on the quantity of complement combining component nitrogen (C′1 N) found in active guinea pig serum.

2. C′1 N was the same when determined with precipitates containing excess antibody or excess antigen.

3. Finely divided specific precipitates took up the complement combining component (C′1) from subsequently added guinea pig serum almost as well as specific precipitates formed in the presence of complement.

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