QUANTITATIVE CHEMICAL STUDIES ON COMPLEMENT OR ALEXIN

IV. Addition of Human Complement to Specific Precipitates*

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The relative titers of the four recognized components of complement vary greatly in the sera of different animals. Hence the "titer" of the complement of a given species is limited by the quantity of component present in lowest titer and comparative titrations of the complement of different species, carried out in the usual manner, may yield data which are not comparable if two different components limit the titer. Hegedüs and Greiner (1) again demonstrated this by separate titration of the four components of the complement of numerous animals and concluded that the low titers generally obtained with unfractionated human complement (C′), for example, were caused by a shortage of second component, or "endpiece" (C′2). The "titer" did not exceed that of C′2 although the human sera tested contained as much first component (C′1, combining component, or "midpiece") as guinea pig serum, the usual standard in complement studies. In human sera, then, complement titrations carried out in the usual way give the "endpiece" or second component (C′2) titer and not that of C′1, the usual limiting component of guinea pig serum with which comparison is ordinarily made. This may be a reason why "titers" of human complement have failed to reflect clearly conditions of health and disease, for the behavior of the first component, or combining component, C′1, which is believed to be chiefly taken up in complement fixation and bacterial lysis as well as in hemolysis, would seem of greater interest.

Complement titrations may, however, be carried out in human sera so as to yield information as to the C′1 titer, either by addition of crude guinea pig "endpiece" (1), or, as in the present studies, by addition of a small quantity of supernatant from a complement-fixing specific precipitate in guinea pig serum. While the results of such titrations should provide data comparable with those given by guinea pig sera and might have considerable value in

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clinical medicine, the figures so obtained would still be purely relative and unrelated to the actual quantities of the components involved. According to Hegedüs and Greiner, the "titer" of guinea pig C'1 is lower than that of the other components, whereas Pillemer, Ecker, and their collaborators (2) have actually isolated more than twice as much C'1 from guinea pig serum as C'2 and C'4 combined. If these represent the actual proportions of the components in the original complement, considerably less C'2 and C'4 than C'1 are necessary for hemolysis and the "titers" are in inverse ratio to the actual quantities of these components on a weight basis. Since it is now possible to measure the combining component or components of guinea pig complement in weight units, or absolute, rather than relative terms (3–5), it seemed desirable to extend the method, if possible, to human complement or alexin. The present report deals mainly with experiments toward this end.

EXPERIMENTAL

Materials and Methods

Fresh human serum (complement, C') was obtained, with the cooperation of Dr. W. H. Gillespie, Dr. A. B. Gutman, and Dr. C. A. Ragan, Jr., either from normal volunteers (numbered samples) or from patients with hypertension or cardiac disease in whom bleeding seemed desirable (lettered samples). Inactivated complement (iC') was prepared by keeping portions of the sera at 56°C. (thermometer in the serum) for 50 minutes (3). C' and iC' were treated with 1 per cent by volume of 1 per cent merthiolate[1] solution, allowed to stand overnight, and centrifuged again in the cold[2] before use.

The immune systems used were (1) egg albumin (Ea) and anti-egg albumin rabbit serum, (2) the specific polysaccharide of Type III pneumococcus (S III) and anti-pneumococcus (anti-Pn) Type III rabbit serum, and (3) S II and anti-Pn II rabbit serum. The antisera were not inactivated as they were no longer fresh, and were, in addition, used at dilutions at which their complement nitrogen content could not introduce a measurable error.

Precipitin estimations were carried out quantitatively as described in numerous papers (for instance 3, 6) with accurately measured volumes of serum and antigen dilutions. Analyses were usually run in triplicate and the specific precipitates were washed three times instead of twice (3, 5). Determinations 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 the nitrogen of complement combining component or components (provisionally designated C'1 N in (3–5)), while the saline series served as a control for the completeness of inactivation of iC'. In all instances the rabbit antiserum was thoroughly mixed with C' or iC' before addition of antigen.

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[1] Manufactured by Eli Lilly and Company, Indianapolis.

[2] In an International Equipment Company refrigerated centrifuge.

Hemolytic "units" were measured as in (3) both in the original sera and in the supernatants.

In order to supply the missing components for the fortification of human complement in the hemolytic titration fresh supernatants from the above C′ series were used, or, if these were not available, supernatants were quickly prepared as follows: For each milliliter of guinea pig serum specific precipitate was formed with about 0.02 mg. of egg albumin nitrogen and 0.1 to 0.2 mg. of rabbit anti-egg albumin nitrogen, or with similar amounts of Type II or Type III pneumococcus specific polysaccharide and homologous rabbit antibody. These quantities of specific precipitate have been shown to remove C′1, or the combining component(s), from complement. The mixture was centrifuged after the precipitate flocked and the supernatant was used as indicated.

TABLE I

Human Complement Titrations with and without Reinforcement

Human serum and dilution used	Guinea pig serum supernatant	Hemolysis by quantity of human serum dilution used:							
		0.05 ml.	0.075 ml.	0.1 ml.	0.15 ml.	0.2 ml.	0.25 ml.	0.3 ml.	0.4 ml.
B_1 1:30	−						ac	c	
" "	+			+++±	c				
C, 1:10	−					+++±	c		
" 1:30	+			+	+++	c			
E, 1:10	−	++		+++±	c	c			
" 1:40	+			+++	ac	c			
2_0, 1:25	−					+	+++	+++±	
" "	+	+++	ac	c					
13_0, 1:10	−					+++		ac	
" 1:30	+	+++	ac	c					
17_0, 1:10	−				ac	c	c		
" 1:30	+		ac	c	c				
$28–32_0$, 1:40	−			0		ac			c
" 1:320	+			0		++			c

Experiments

For the typical titrations given in Table I 0.2 ml. of hemolytic system (equal volumes of 5 per cent sheep red cell suspension and a dilution of hemolysin containing 2 or 4 "units") was added to each tube, followed by 0.05 ml. of the guinea pig serum supernatant, made up to double the original serum volume. Varying volumes of diluted human serum were then added, followed by saline to a total of 0.5 or 0.6

ml. The usual controls were run, including one with 0.1 ml. of guinea pig supernatant with saline and hemolytic system. This was negative in every instance. For each serum the first line in the protocol gives the titration without supplement, while the second line indicates the result of addition of the missing component or components.

Lot D.—In an earlier experiment C′ and iC′ blanks with saline alone showed no less N than corresponding blanks with antigen and serum alone. Saline blanks were therefore dispensed with. As neutralization of guinea pig C′ and iC′ had been found unnecessary, this step was also omitted with the human sera and earlier protocols are not given. pH of C′, 7.38; pH of iC′, (after 50 minutes at 56°C.), 8.21. Diluted anti-Pn III rabbit serum pool, B 40, 60 used. S III, 0.04 mg. per ml. C′2 "titer" determined 1 week later after storage in CO_2 snow, 70 units per ml.; C′1 "titer" (with guinea pig serum supernatant), 120 units. Tubes held at 19–21°C. for 2.5 hours, overnight in ice box before centrifuging.

No. of tubes	1	1	1	1	3	3	3	3	3	3
C′, *ml.*	6.0	6.0						2.0	4.0	6.0
iC′, *ml.*			6.0	5.5		2.0	6.0			
Serum dilution, *ml.*	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
Saline, *ml.*	1	1	1	1	6	4		4	3	1
N precipitated, *mg.*	0.012	0.012	0.018	0.024*	0.444 0.448 0.446	0.470 0.476 0.482	0.492 0.500 0.492	0.556 0.568 0.554	0.624 0.590 0.610	0.616 0.612 0.634
Mean	0.012		0.021		0.446	0.476	0.495	0.559	0.608	0.621
Subtraction of blank						0.007‡	0.021	0.004‡	0.008‡	0.012
Specific N precipitated, *mg.*						0.469	0.474	0.555	0.600	0.609
Subtraction of iC′ value								0.469	0.472§	0.474
C′ N precipitated, *mg.*								0.09	0.13	0.14
C′ N precipitated per ml. C′ taken								0.045	0.033	0.023

* Calculated to 6.0 ml.

‡ Aliquot portion of blank on 6.0 ml.

§ Mean of the two iC′ series values.

Hemolytic units left in each 2, 4, 6 ml. C′ supernatant respectively, $<<40$, <80, $<<160$.

Data obtained with another portion (D′) of the same lot kept frozen a week longer, and then analyzed, are given in Fig. 1.

Lot E.—Anti-Ea rabbit serum pool *d*, diluted to 3.5 volumes, (0.4 mg. anti-Ea N per ml.) and Ea, 0.04 mg. N per ml. were used as the immune system. C′2 "titer" of lot E, 70 units; C′1 "titer" (with guinea pig serum supernatant), 230 units. Tubes centrifuged after 3 hours at 20–21°C.

Portions of lot E, active and inactivated as in the previous section, were first treated with anti-Pn horse specific precipitate before addition of the rabbit system, as had been done in the study of guinea pig complement (3). The anti-Pn VIII horse serum H644 used had been precipitated with S III in another connection (7)

No. of tubes	1	1	1	1	3	3	3	3	3	3
C′, *ml.*	3.0	3.0					1.5	2.5	4.0	6.0
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
Ea dilution, *ml.*		0.5		0.5	1.0	1.0	1.0	1.0	1.0	1.0
Saline, *ml.*	1	0.5	1	0.5	3					1
N precipitated, *mg.*	0	(Lost)	0.010	0	0.394 0.386 0.396	0.404 0.396 0.384	0.460 0.464 0.464	0.490 0.484 0.476	0.506 0.512 0.500	0.534 0.524 0.540
Mean	0		0.005		0.392	0.395	0.463	0.483	0.506	0.533
Subtraction of blank						0.005	0	0	0	0
Specific N precipitated, *mg.*						0.390	0.463	0.483	0.506	0.533
Subtraction of iC′ value*							0.390	0.390	0.390	0.390
C′ N precipitated, *mg.*							0.07	0.09	0.12	0.14
C′ N precipitated, per ml. C′ taken							0.047	0.036	0.030	0.023

* Since 3.0 ml. iC′ gave the same value as the salt control series it is assumed that amounts of iC′ corresponding to the other volumes of C′ used would also have given the same value.

Hemolytic units left in each 1.5, 2.5, 4, 6 ml. supernatant, respectively, <7, <9, <12, 20.

and had been diluted to an anti-S VIII content of 0.65 mg. N per ml. After the reaction components were mixed, the tubes were allowed to stand 1 hour at 21°C.

C′, *ml.*	5	5	30		
iC′, *ml.*				30	
Saline, *ml.*			0.5	0.5	30.5
Anti-Pn VIII horse serum, *ml.*	0.8		5.0	5.0	5.0
S VIII, 0.17 mg. per ml., *ml.*		0.2	1.0	1.0	1.0
N precipitated from aliquot, *mg.*	0.016*	0.016*	0.538 0.538 0.536	0.548 0.550 0.554	0.518 0.524 0.520
Mean	0.016		0.537	0.551	0.521
Total N precipitated, *mg.*			2.15	2.20	2.08
Subtraction of blank, *mg.*			0.10	0.10‡	
Specific N precipitated, *mg.*			2.05	2.10	2.08

* Entire precipitate from blanks used for analysis.

‡ C′ and iC′ blanks in the preceding experiment with serum E were approximately the same. Mean blank (first 2 columns) × 6 used.

and were centrifuged in the cold. All supernatants were recentrifuged and the precipitates were washed 3 times with 10 ml. of chilled saline, dissolved in alkali, and rinsed into 20 ml. volumetric flasks. Aliquot portions of 5.0 ml. were analyzed for N.

The recentrifuged C′, iC′, and saline supernatants from the above reaction were set up with the same Ea and rabbit anti-Ea dilutions used in the earlier experiment

on untreated human serum E. 6 ml. of each supernatant represented 4.9 ml. of the original C′, iC′, or saline.

No. of tubes	1	1	1	1	3	3	3
C′ supernatant, *ml.*	6.0	6.0					6.0
iC′ " *ml.*			6.0	6.0		6.0	
Saline " *ml.*					6.0		
Anti-Ea serum, *ml.*	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.*	1	1	1	1			
N precipitated, *mg.*	0.024	0.008	Lost	0.014	0.392 0.386 0.382	0.410 0.406 0.396	0.502 0.496 0.524
Mean	0.016		0.014		0.387	0.404	0.507
Subtraction of blank, *mg.*						0.014	0.016
Specific N precipitated, *mg.*						0.390	0.491
Subtraction of iC′ value							0.390
C′ N precipitated, *mg.*							0.10

Hemolytic units taken, per 4.9 ml. original C′, 1125.
" " in C′ horse serum blank, " " 930.
" " " C′-S VIII " " " 1030.
" " " C′-S VIII-anti-Pn VIII supernatant, 960.
" " " final supernatant, <16.

Because of the apparent solubility effect in other experiments (see also Fig. 1) a run was made with 30 ml. of C′ with the rabbit system. It was thought that at this volume the inhibiting effect of C′ excess might be great enough to mask the addition of C′ N and give N figures like those with S VIII and horse antiserum in spite of fixation of the C′ present. A portion of lot H of human serum (C′1 "titer," 200 units per ml.) was used as no more of lot E was available. Anti-Pn-III rabbit serum B 40, diluted to 9 volumes. S III, 0.19 mg. per ml. Tubes allowed to stand at room temperature for 3 hours, in ice box overnight. Analyses run as in protocol 2, lot E.

C′, *ml.*	10	30.0	
Saline, *ml.*	2		30
Anti-Pn III rabbit serum, *ml.*		5.0	5.0
S III solution, *ml.*		1.0	1.0
N precipitated from aliquot, *mg.*	0.032	0.750 0.756 0.754	0.570 0.574 0.566
Mean	0.032	0.753	0.570
Total N precipitated, *mg.*		3.01	2.28
Subtraction of blank, *mg.*		0.10	
Specific N precipitated, *mg.*		2.91	2.28

The difference between the C′ and saline series, 0.63 mg. of N, indicates that the apparent solubility effect does not account for the values found with S VIII and anti-Pn VIII horse serum.

Lot G.′—A comparison was made of the C′ N removed from this serum by two different immune systems, Ea-anti-Ea, and S II-anti-Pn II. The former was that used in the preceding instance. Ea solution, 0.045 mg. N per ml. Anti-Pn II rabbit serum 580, diluted to 4 volumes with saline. S II, 0.1 mg. per ml. C′2 "titer" of lot G′, 80 units; C′1 "titer" (with guinea pig serum supernatant), 800 units.

No. of tubes	1	1	1	3	3	3	2
C′, *ml.*	1.0	2.0	2.0			2.0	2.0
Anti-Ea dilution, *ml.*				1.0			1.0
Ea dilution, *ml.*	0.2			1.0			1.0
Anti-Pn II dilution, *ml.*		0.5			1.0	1.0	
S II dilution, *ml.*			0.5		1.0	1.0	
Saline, *ml.*	0.2	0.5	0.5	2	2		
N precipitated, *mg.*	0.006	0.006	0.002	0.448 0.438 Lost	0.120 0.146 0.150	0.198 0.198 0.210	0.528 0.526
Mean	0.012*	0.004		0.443	0.139	0.202	0.527
Subtraction of blank						0.004	0.012
Specific N precipitated, *mg.*						0.198	0.515
Subtraction of saline blank value						0.139	0.443
C′ N precipitated, *mg.*						0.06	0.07

* Calculated to 2.0 ml.

Hemolytic units left in each Ea system supernatant, $\ll 13$; in Pn II system supernatant, 20.

Lot 19_2.—C′2 "titer," <75 units; C′1 "titer," 400 units per ml. This human serum, containing antibodies to Type II pneumococcus, was preserved with 1:10 000 merthiolate and handled and analyzed so as to favor sterility. Estimation of C′ N on 2.5 ml. samples with Ea-anti-Ea as in previous examples gave 0.08 mg. C′ removal was practically complete. 4.5 ml. portions of the supernatants from the C′ analysis, corresponding to 2.5 ml. of original serum, were each mixed with a solution of 0.06 mg. of S II and allowed to stand in the ice box for 5 days, since in this and other instances of the analysis of weak human sera precipitation increased visibly during the first few days. Similar analyses were made with fresh serum 19_2 containing C′. Blanks were also run and deducted.

Specific N from 2.5 ml. containing C′, 0.117 mg.
Anti-S II N from equivalent volume "decomplemented" serum, 0.090 mg.
Human C′ N removed by S II-human anti-S II, 0.027 mg.

C′1 units in supernatant, calculated to original volume, 160 per ml.

In this instance the human specific precipitate took up 30 per cent of its weight of C′ and removed more than one-half of the number of C′1 units present.

DISCUSSION

It was shown in the introduction and in Table I that titration of human complement in the usual way does not yield the same information as with guinea pig complement and a new titration procedure for overcoming this difficulty was proposed. It is evident from the Experimental part, however, that one need not remain content with relative figures, and that the quantitative estimation of complement combining component or components in absolute terms, or weight units (3) is applicable without modification to human sera.

The amounts of complement nitrogen added to specific precipitates in fresh human serum are of the same order of magnitude as in guinea pig serum, but there seems to be little relation to the hemolytic titers of the various samples, whether these titers were obtained in the usual way or with added guinea pig serum supernatant. The reason for this is not clear but may be related to the growing mass of evidence that other components of C′ as well as C′1 are taken up.

When the present method for complement estimation (3) was proposed, it was made clear that the nitrogen determined was that of complement as defined by Muir (8) and that the designation C′1 N was a provisional one in accord with current belief that the combining component, or components, consisted mainly of "midpiece" or C′1 (9, 10). The inadequacy of this belief has often been maintained (11). It has now been shown in new work by Ecker and Pillemer (12) and by our own studies, which it is hoped soon to publish, that other complement components also add to specific precipitates. The quantitative method therefore remains, as before, a valid one for the estimation of complement or, more strictly, complement combining components in weight units.

Fig. 1 shows that the apparent solubility effect is also present as in guinea pig complement. If the lines in the figure are extrapolated roughly to zero volume they lead to the conclusion that the sera analyzed contain between 0.03 and 0.05 mg. per ml. of nitrogen due to complement combining component or components. This might provisionally be taken as the normal range of fluctuation for human sera. Many other samples, both from normals and from cardiac and hypertensive cases, have fallen within the same limits. These are not shown in the chart, partly to avoid overcrowding, and partly because many of the analyses were carried out with a single volume of serum.

It is also shown in the experiment on lot G′ that the quantity of C′ N found does not depend on the immune system used and is like that found in other sera with S III-anti-S III.[3] The second portion of the experiment on lot E, however, shows that human complement does not add to a specific precipitate from anti-

[3] Irregular results with the S I-anti-Pn I rabbit serum system in guinea pig and human sera are under investigation.

pneumococcus horse serum, and that in this respect, also, it resembles guinea pig complement (3, 13). The experiment shows, further, that the nitrogen ascribed to C′ cannot be due to insoluble or easily adsorbed material other than C′, since no more nitrogen was carried down by quite adequate amounts of the horse specific precipitate from C′ than from iC′ or saline. Moreover the supernatant showed very nearly the same quantity of C′1 N, when subsequently treated with egg albumin-rabbit anti-egg albumin as did the untreated portion of lot E.

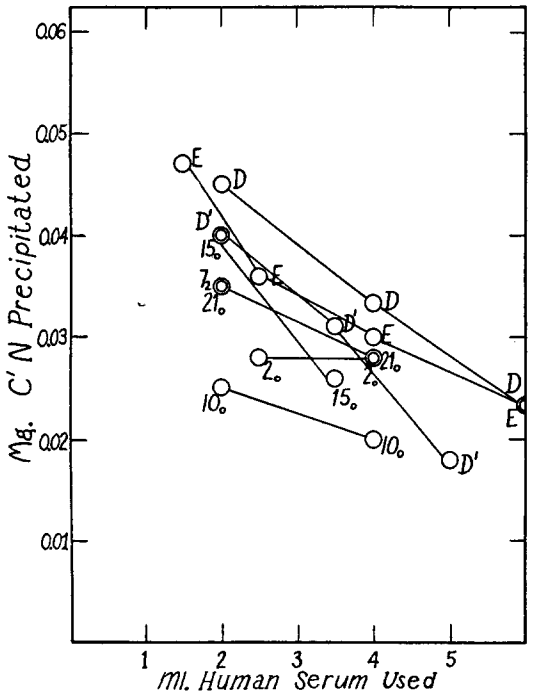

FIG. 1

Initial experiments, as with lot 19_2, indicate that human complement may be fixed in amounts comparable to those of guinea pig complement by human antipneumococcus serum in the reaction with homologous polysaccharide. This is perhaps surprising in view of Horsfall and Goodner's observation (14) that guinea pig complement is not fixed by human sera in reacting with encapsulated pneumococci.[4] The fixation of appreciable amounts of

[4] Fixation of human complement by human immune sera is evidently subject to unknown factors. Thus far 4 sera have shown precipitation of more specific N in the presence of human C′ than in its absence, but in only 2 of these was the decrease in "titer" checked. Two other sera, on the other hand, showed no difference in specific N and no decrease in "titer."

human C′ N in the S-human-anti-Pn system complicates the interpretation of these and other precipitin reactions in human sera. It is quite likely that small quantities of antibody, such as might occur during convalescence or after active immunization, could easily appear to be doubled (*cf.* 4) by the C′ N fixed during combination of the antibody with antigen and thus indicate too high an antibody content. It is also possible that inactivation of human sera for a period long enough to abolish fixation of C′ N (50 minutes at 56°C.) would damage small amounts of antibody. Therefore it appears best, while these possibilities are being investigated on a large number of sera, to remove C′ by the method used in the experiments referred to, namely, by absorption with adequate amounts of an unrelated precipitating system, such as Ea-rabbit anti-Ea. Attention is also called to the length of time (4 to 5 days at 0°C.) required for complete separation of small quantities of specific precipitate from human sera, a period which seems to be lengthened, rather than diminished, by the coprecipitation of active complement. As is stated in (3) flocculation even of relatively large amounts of specific precipitate from rabbit antibody is delayed by guinea pig C′. The same observation was made throughout the experiments with human C′ and in many instances the delay in flocculation was even more marked than with guinea pig C′.

SUMMARY

1. A modified method is given for the titration of human complement so that C′1 titers are measured, as in guinea pig serum, instead of the C′2 titers yielded by the usual titration.

2. The measurement of complement combining component or components in weight units, instead of relative terms, is carried out as in guinea pig serum and leads to similar values, 0.03 to 0.05 mg. of C′ N per ml. of human serum.

3. Other similarities in human and guinea pig complements are noted and discussed.

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