A COMPARISON OF HUMAN AND GUINEA PIG COMPLEMENTS AND THEIR COMPONENT FRACTIONS*

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One approach to the problem of the biological function of complement is the comparative study of this strange and unstable complex as it occurs in the sera of different species of animals. The first of two recent studies along these lines was that of Hegedüs and Greiner (1). The reasonable hypothesis was propounded that the titer of a complement is limited by the component present in lowest titer, and that each of the four components might be titrated independently by addition of the other three components in excess, so as to make the component in question the one present in lowest titer. Required reagents were prepared by standard methods from guinea pig complement, apparently in the firm conviction that each component of this complement was wholly equivalent in function to the corresponding component of the complement of the animal species being tested, and *vice versa*. The validity of this assumption was denied by Ecker, Pillemer, and Seifter in a comparative study of human and guinea pig complements (2) because certain substitutions were not found "effective."

The complements of these two species have been under investigation for several years in the laboratories of the Presbyterian Hospital (3–8, and unpublished studies). While there had been no reason to question the assumptions underlying the work of Hegedüs and Greiner, doubts had arisen as to the adequacy of their technique for measuring the various components and their conclusions as to the relative quantities of the components in guinea pig complement had not been confirmed. It also became evident that experimental conditions could be found under which the components of human and guinea pig complements are mutually substitutive[1] and that existing methods for the titration of the components of complement required revision in order to render them suited to their purpose. It was therefore decided to submit evidence on these matters already in hand and to extend the study in order to learn more

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[1] Ecker, Pillemer, and Seifter have now independently concluded that the components are equivalent (private communication from Dr. Ecker).

of the part played by each component in the fixation of complement in general and the uptake of complement nitrogen by specific precipitates in particular, problems still in large measure unsolved in spite of early (9, 10) and more recent studies (8, 11–13).

Accordingly, estimations were made of the titers of the components present in "midpiece," "endpiece," and in the so called "specifically inactivated" complements (11) prepared by different methods, since this was a prerequisite to an understanding of these reagents and to their use in quantities adequate to provide, in optimal excess, the components desired.

The greatest care has been taken in the present studies to use each reagent well below its anticomplementary range. Emphasis on this rudimentary precaution appears indicated by the lack of any positive evidence that it was observed in numerous recent investigations.

The data herein reported are given in some detail since fundamental principles and procedures are involved. It is felt that due consideration of the data and discussion will aid in dispelling some of the confusion surrounding the subject of the components of complement.

EXPERIMENTAL

Methods and Materials

Complement.—Blood from healthy guinea pigs was obtained by cardiac puncture without anesthesia. Human blood was taken by venous puncture. After about 1 hour at room temperature, the serum was drawn off, centrifuged, and used either on the same day or stored, tightly-stoppered, in a dry-ice freezer until needed. Complement preserved in this way did not lose activity even after several months' storage, although prolonged exposure to CO_2 sometimes lowered the pH, necessitating addition of 0.1 N $NaHCO_3$ to pH 7.5 or 8.

Sheep Red Cells and Hemolysin.—Defibrinated blood was obtained once a week, alternately from each of two sheep. The red cells were washed 4 times, and a 5 per cent suspension containing about 125 million erythrocytes per 0.1 ml. was sensitized with an equal volume of a rabbit anti-sheep cell hemolysin dilution containing about 4 "units" (estimated in the usual way with excess complement) per 0.1 ml. 0.2 ml. of sensitized red cell suspension was employed in each test; that is, roughly 125 million red cells sensitized with 4 "units" of hemolysin.

Hemolytic Titer.—This was estimated by addition of varying quantities of a dilution of complement, reagent, or component to 0.2 ml. portions of the sensitized red cell suspension and addition of saline, if required, to make a total volume of 0.6 ml. In instances in which relatively large volumes of reagent were necessary volumes up to 0.8 ml. could be used with little influence on the end result. The tubes containing the mixtures were incubated in a water bath at 37–38°C. for 30 minutes, with shaking every 10 minutes. The number of hemolytic units per milliliter of undiluted serum was calculated by dividing the dilution employed by the smallest volume giving complete hemolysis. For the estimation of single complement components in whole, fractionated, or "specifically inactivated" complements, titrations were carried out in the presence of a constant optimal quantity of the proper reagent as described below.

Preparation of the Reagents. A. "Midpiece" (M) and "Endpiece" (E).—Fractionation of complement was carried out by dialysis or dilution following Ferrata (14), Liefmann (15), and later workers (2, 11). The dilution method took less time, and sometimes gave better recoveries of C'2[2] and C'4 in E.

1. Dilution Method.—To 10 volumes of chilled M/200 KH_2PO_4 solution, 1 volume of chilled guinea pig (g.p.) or human (hu) serum was added slowly, with constant mixing in the cold. The pH of the mixture approached neutrality. After 20 to 30 minutes at 0°C., the precipitate was centrifuged off in the cold, separated as completely as possible from the supernatant and, in the case of g.p. C',[2] washed with phosphate solution of approximately the same ionic strength and pH as the final value. The precipitate from hu C' was not washed since hu serum contains much less C'2. The preciptate, or "midpiece," was dissolved in saline, usually with addition of 0.1 N $NaHCO_3$ until neutral, and made up to 5 times original serum volume for further dilution as needed. The original supernate, "endpiece," was made isotonic with 10 per cent NaCl and neutralized with 0.1 N $NaHCO_3$ as soon as possible, since C'2 is unstable below pH 6.5 (16, 17). The final dilution was 1:12. In the fractionation of g.p. C', the CO_2 dilution method (15) was used in numerous instances as well.

2. Dialysis Method.—Directions in (2) were followed with several modifications. The period of dialysis was shortened to 6 hours when 5 ml. portions of serum were used, to avoid deterioration of components unstable in the acid pH range. This was accomplished by increasing the dialyzing surface by placing a Pyrex test tube inside the cellophane bag to force the serum into the resulting annular space and by changing the outside buffer solution every 2 hours and mixing frequently. The final dilutions were 1:5 for M and 1:10 for E.

B. Sera Lacking Either C'3 or C'4.—The preparation of human or guinea pig complement lacking in one of the two relatively heat-stable components was carried out according to (2, 18, 19) with little modification, confirmation being obtained of the necessity for inactivating C'3 with "zymosan" (18) at slightly alkaline reaction. About 15 times as much zymosan was required for the inactivation of g.p. C'3 as for hu C'3. The optimal period for the C'4 inactivation with ammonia was 1 hour.

C. Heat-Inactivated Serum.—Hu and g.p. sera were heated at 56°C. for 20 minutes, this being sufficiently long to inactivate C'1 and 2 and less destructive for C'3 and 4 than 30 minutes' heating. In every instance in which this reagent was used, 0.05 or 0.1 ml. of 1:5 dilution was employed.

D. Sera Possessing Activity of a Single Complement Component.—By treating heated hu and g.p. sera with either ammonia or zymosan (2, 18), reagents were obtained with the single reactivity of C'3 or C'4. When C'1 alone was wanted, "midpiece" from sheep serum sufficed (*cf.* 1).

Titration of the Four Components of Complement

A reagent for the titration of a given component should not contain the component to be titrated, but should contain an excess (*i.e.*, > 1 unit per dose employed) of each of the remaining components. Four reagents are required (*cf.* 1): reagent 1, containing C'2, 3, 4, for the titration of C'1; reagent 2, containing C'1, 3, 4, for the titration of C'2; reagent 3 with C'1, 2, 4, for the titration of C'3; and reagent 4, containing C'1, 2, 3, for the titration of C'4. A

[2] In agreement with Ecker and Pillemer complement is designated C' and the components C'1, C'2, C'3, and C'4.

reagent should produce no lysis of sensitized red cells when used alone in several times the quantity employed in actual titrations.

Reagent 1 was prepared by combination of E, which furnishes C'2 and some C'3 and C'4, with heated serum which supplies extra C'3 and C'4. Reagent 2 consisted of M plus heated serum. In each instance the heated serum was added separately to the types in which the tests were carried out. Zymosan-treated C' (2, 18) was employed as reagent 3, while ammonia- or hydrazine-treated C' served as reagent 4.

For purposes of control, the adequacy of each reagent should be established by experiment, a condition which was not adequately met in (1, 2, etc.) This requires testing each reagent (R) with every other reagent, as follows: (*a*) R1 + R2; (*b*) R1 + R3; (*c*) R1 + R4; (*d*) R2 + R3; (*e*) R2 + R4; (*f*) R3 + R4. If complete hemolysis of added sensitized red cells follows, the first test demonstrates the presence of 1 or more units of C'2 in the amount of R1 used, and one or more units of C'1 in R2. Similarly, the second test establishes the presence, in adequate amount, of C'3 in R1 and C'1 in R3. If all tests result in complete hemolysis, it is certain that each of the four reagents actually contains the three components which it is designed to supply. While it is desirable to perform a complete test with every set of reagents, this was not always done in the titrations described below, in some of which control tests for critical components, only, were included.

Below the anticomplementary limit (see next section), the quantity of a reagent should be chosen in a range in which the titer of the component remains independent of the amount of reagent employed. The complete quantitative composition of the reagents was not established in every experiment. It is, however, possible to give the composition of average preparations of each reagent from data gathered in the course of the work, as noted in the subsequent sections. For example, "endpiece" (E), which is used in R1, showed the following average composition:—

Source and method of preparation	C'2 titer	C'3 titer	C'4 titer
	units/ml.	*units/ml.*	*units/ml.*
Hu, dialysis	110	35	2400
" dilution	115	45	2400
G.p., dialysis	140	60	3500
" dilution	220	60	2100

It is evident that E is generally deficient in C'3, since less than 100 units are present per milliliter and 0.1 ml. of a 1/10 dilution (the amount generally employed) contains < 1 unit of C'3. The addition of heated complement, by supplying C'3, serves to remedy the deficiency and is especially necessary with

hu E. G.p. E + heated g.p. serum yielded the best R1, since g.p. E contains more C'2 than hu E, and heated g.p. serum is not anticomplementary and supplies more C'3 than heated hu serum.

Guinea pig C' inactivated for 20 minutes at 56°C. usually titered 100 to 300 units of C'3 and about 500 to 2000 units of C'4. The C'4 titer of heated human serum was similar, but the C'3 content was very low.

The average composition of "midpiece" (M) follows:—

Source and method of preparation	C'1 titer	C'3 titer	C'4 titer
	units/ml.	*units/ml.*	*units/ml.*
Hu, dialysis	1000	90	350
" dilution	1000	40	130
G.p., dialysis	350	120	100
" dilution		100	

Again it is seen that M should be combined with heated complement for use as R2, owing to its deficiency of C'3 and often of C'4 as well. This applies especially to hu M, which was preferred for R1 since it is not anticomplementary. G.p. M, when freshly prepared, should not be anticomplementary, but often becomes so if kept in solution, or if made alkaline.

Average Composition of Zymosan-Treated Complement

Source	C'1 titer	C'2 titer	C'4 titer
	units/ml.	*units/ml.*	*units/ml.*
Hu	2000	100	2000
G.p.	1000	260	2000

If at least 0.02 ml. (0.1 ml. of 1:5 dilution) is used, this reagent functions as R3.

Average Composition of Ammonia-Treated Complement

Source	C'1 titer	C'2 titer	C'3 titer
	units/ml.	*units/ml.*	*units/ml.*
Hu	2000		
G.p.	1200	300	100

Anticomplementary Action of the Reagents.—The anticomplementary effect of g.p. "midpiece", when encountered, was found to be somewhat greater toward homologous C' than hu C':—

G.p. M added		G.p. C′ (1:40)			Hu C′ (1:10)		
Dilution*	Ml.						
		ml., 0.05		0.18	0.05		0.1
1:5	0	−		c	++		c
	0.15	−		+	−		+
		ml., 0.08	0.1	0.15	0.08	0.1	0.15
	0	+	++±	c	+	+++	c
1:100	0.15	−	±	+±	±	+	c
	0.15‡	+++	c	c	+++	c	c
		ml., 0.05	0.08	0.13	0.05	0.08	0.13
	0	±	+++±	c	±	+++	c
1:50	0.1	−	+	+++	−	±	c
	0.3	−	+	++	−	−	±
		ml., 0.05	0.1	0.2	0.05	0.1	0.2
	0	−	+++±	c		++±	c
1:5§	0.05	+	+++	c	+	ac	c
	0.05‖	+++±	c	c	+++±	c	c

In this and subsequent protocols − to ++ to c indicates degree of hemolysis. +++± = a few red cells left; ac = faint haze; c = complete, crystal-clear hemolysis.

* Based on volume of original g.p. serum used for preparation of reagent. A different preparation of M was used in each of the experiments.

‡ Plus 0.05 ml. of 1:5 dilution of heated g.p. serum, to complete R2.

§ Freshly prepared by dialysis; neutralized, but not made alkaline.

‖ Plus 0.08 ml. of 1:5 heated g.p. serum.

Addition of heated g.p. serum greatly reduced this anticomplementary action of g.p., hu, and sheep M, the only ones tested.

Hu M is seldom anticomplementary toward hu C′ but may be toward g.p. C′:—

Hu M added		Hu C′ (1:10)			G.p. C′ (1:40)		
Dilution	Ml.						
		ml., 0.08	0.1	0.13	0.08	0.1	0.13
	0	++	ac	c	++±	c	c
1:5	0.05	+++	ac	c	++	+++	c
	0.05*	c	c	c	ac	c	c
	0	+	+++		+		c
1:5	0.05	+	+++		±		++±
	0.05*	+±	ac		c		c

* Plus 0.05 ml. 1:5 heated g.p. serum to complete R2.

TABLE I

Titration of C'1 in Hu and G.P. Complements

Human				Guinea pig			
Sample	Dilution	Reagent	C'1 titer	Sample	Dilution	Reagent	C'1 titer
	1 to:		*units/ml. undil. C'*		1 to:		*units/ml. undil. C'*
Pool 1	400	G.p. E + heated g.p. serum*	4000	Individual, 1	200	G.p. E	1300
				" 1	200	R1	1300
" 2	300	G.p. E	4000	Pool 1	200	G.p. E	1300
" 2	300	R1	4000	" 1	200	R1	2000
" 3	300	G.p. E	2000	" 2	300	G.p. E	1200
" 3	300	R1	3000	" 2	300	R1	1500
" 4	200	"	2000	" 3	400	G.p. E	4000
				" 3	400	R1	4000
Individual, 1	200	R1	2700				
				" 4	200	G.p. E	1000
" 2	300	Hu E	2000	" 4	200	R1	2000
" 2	300	" " + heated hu serum	850				
				" 5	200	G.p. E	2700
" 2	300	Hu E + heated g.p. serum	2000	" 5	200	R1	2700
				" 6	350	"	6000
" 3	500	G.p. E	6700				
" 3	500	R1	6700	" 7	120	"	1000
" 4	300	"	4000	" 8	400	Hu E + heated g.p. serum	1300
" 5	500	"	5000				
" 6	300	G.p. E	3000	" 8	400	R1	1300
" 7	300	R1	4000	" 9	300	"	1500
" 8	400	"	2000	" 10	200	"	700
" 8	400	Hu E + heated g.p. serum	2000				
" 9	200	R1	1000				

* 0.05 ml. of 1:5 heated g.p. serum to complete R1.

Hu and g.p. C' treated with zymosan were not anticomplementary nor was ammonia-treated g.p. serum. Hu serum treated with ammonia was always

anticomplementary toward g.p. serum, but not against hu serum, g.p. E, or heated g.p. serum.

Titration of the Four Components in Human and Guinea Pig Complements and the Reagents Prepared from These.—With the limits established within which the various reagents might be used, titration of the four components was now possible.

1. Titration of First Component, C'1.—Complement dilutions most suitable for titration of C'1 were found to be between 1:200 and 1:500. Increasing amounts were set up with an amount of reagent 1, usually between 0.2 to 0.5 ml., previously found to contain an optimal excess of C'2, 3, and 4. Reagent 1, prepared by use of a volume of g.p. E such as given above, and 0.05 ml. of 1:5 heated g.p. serum, was found preferable to that from hu E, as with the latter, amounts up to 1 ml. were sometimes required, and anticomplementary activity was also encountered. However, as noted at the bottom of Table I, comparable C'1 titers could be obtained with either reagent under favorable conditions (freshly prepared hu E). As prepared and used, g.p. R1 usually contained about 3 units of C'2, 3 of C'3, and 50 to 100 of C'4. C'1 titrations of hu and g.p.C' are reported in Table I. Numerous other samples were tested, with results in the same range.

For the titration of C'1 in "midpiece" a dilution of about 1:100 was found convenient. Again, g.p. R1 was the reagent of choice although similar results could be obtained, but less readily owing to a deficit of C'2, by substitution of hu E for g.p. E. Heated hu serum could also be substituted for heated g.p. serum.

Titration of hu and g.p. M with R1:—

"Midpiece" 1:100	Method of preparation	C'1 titer		Recovery
		Original C'	M	
		units/ml.	*units/ml.*	*per cent*
Hu	Dilution	6700	1600	(25)
"	"	6000	1500	25
"	"	2000	500	25
"	Dialysis, pH 5.4	2000	1000	50
"	" pH 6.7	2000	1000	50
G.p.	Dialysis	1300	350	25
"	"	700	200	30
"	Dilution	1200	700	60

The C'1 titrations carried out on zymosan- and ammonia- or hydrazine-treated sera showed that these inactivation procedures for C'3 and C'4, respectively, are not entirely specific and affect the C'1 content to some extent

TABLE II

Titration of C'2 in Hu and G.P. Complements

Human					Guinea pig				
Sample	Dilution used	Reagent added for C'2 titration only	Titer		Sample	Dilution used	Reagent added for C'2 titration only	Titer	
			Whole C'	C'2				Whole C'	C'2
	1 to:		*units/ml.*	*units/ml.*		1 to:		*units/ml.*	*units/ml.*
Pooled	20	G.p. M* + heated heated g.p. serum	80	160	Pooled	40	Hu M + heated g.p. serum (R2)	400	500‡
		G.p. M* + heated hu serum		80					
"	20	Hu M + heated g.p. serum (R2)	100	130‡	"	40	R2	300	400‡
		Sheep M + heated g.p. serum		130	"	40	"	270	300‡
		G.p. M + heated g.p. serum		130	"	40	"	300	400
"	20	R2	100	200‡	"	40	"	200	400
Individual	20	"	80	200	"	40	"	270	500
"	20	"	100	130	"	40	"	300	500
"	20	"	80	130‡	"	40	"	300	400
"	20	"	100	200‡	"	40	"	500	500
"	20	"	70	80	"	40	"	270	270
"	20	"	80	80	"	40	"	400	400
"	20	"	130	200‡	"	40	"	300	800
"	20	"	130	240‡					
"	20	"	100	100					

* M = "midpiece."

‡ The same titer was obtained by addition of heated g.p. serum without "M," since sufficient C'1 was present in the C' tested.

as well. As will be seen later, the titers of all remaining components are reduced.

Titration of zymosan- and ammonia- (or hydrazine-) treated sera with R1:—

Source	Inactivation by	C'1 titer		Recovery
		in C' used	in reagent	
		units/ml.	*units/ml.*	*per cent*
G.p.	Zymosan	4000	1000	25
Hu	"	6700	5000	75
"	"	2000	1500	75
G.p.	Ammonia	2700	1300	50
"	"	2000	1000	50
"	Hydrazine	2700	1300	50
Hu	Ammonia	2000	2000	100
"	"	1000	400	40

2. Titration of C'2.—R2, for the titration of C'2, was preferably composed of hu M, in amounts of 0.025 to 0.1 ml. of a 1:5 dilution, plus 0.05 ml. of 1:5 heated g.p. serum. As so prepared, the amounts used contained 5 to 20 units of C'1, 2 to 3 of C'3, and 5 to 10 of C'4. R2 prepared with non-anticomplementary lots of g.p. M may also be employed. Titration of C'2 in whole C' may be carried out with heated g.p. serum alone, since C' contains excess C'1. However, for titration of C'2 in E, the complete reagent, R2, is necessary. Representative data are given in Table II. Taken with the other data given, it is evident that C'2 is the component of lowest titer in hu C', and hence the titer-limiting component.

Sheep M plus heated g.p. serum is also useful as R2. Several protocols of titrations of C'2 in "endpieces" are reported below, followed by a summary of numerous titrations in Table III:—

"Endpiece" and dilution used	Reagent: heated g.p. serum plus				C'2 titer
	hu M (R2)		sheep M		
	units of C'1 added	*ml.*	*units of C'1 added*	*ml.*	*units/ml.*
Hu 1	3	0.05			30*
1:12					40
	7	0.10			30*
					40
	3	0.10			120
Hu 2	4.5	0.15			160
1:12	6	0.2			160
	7.5	0.25			120‡
G.p. 1	3	0.05			160
1:48					
	1–7	0.05–0.035			480
G.p. 2			1	0.05	240
1:24			3–5	0.15–0.25	480
			7	0.35	320‡
G.p. 3	2.5–5	0.22–0.44			240
1:48			2.5–5	0.05–0.1	240

* Heated g.p. serum was not added in these two instances.

‡ Shows anticomplementary effect of excess reagent.

TABLE III

Titration of C'2 in "Endpieces" with Reagent 2

Source	Method of preparation	C'2 titer		Recovery
		in C' used	in E	
		units/ml.	*units/ml.*	*per cent*
Hu 1	Dialysis, pH 5.4	130	100	75
" 2	" pH 5.4	130	90	70
" 1	" pH 6.6	130	130	100
" 2	" pH 6.6	130	110	85
" "	Dilution	130	90	70
" 3	"	270	200	75
" 4	"	270	160	60
G.p. 1	Dialysis, pH 6	270	130	50
" 1	Dilution	270	160	60
" 2	"	400	160	40
" 3	"	800	650	80
" 4	"	320	240	75
" 5	Dialysis, pH 6	270	130	50
" 6	"	270	150	55

It is clear from the various combinations used in the titrations for C'1 and C'2 that hu and g.p. C'1 are mutually substitutive and that the same applies to C'2.

After treatment of C' with zymosan or ammonia, or hydrazine, a reduction of C'2 activity occurred along with the elimination of C'3 or C'4. Reduction in C'1 titer has already been noted. From the one instance in which freshly boiled zymosan was used, the value of this precaution (18) seems evident. The data follow:—

Effect of Zymosan or NH_3 Treatment on C'2 Titer of C'

Source	Inactivation by	C'2 titer		Recovery
		in C' used	in reagent	
		units/ml.	*units/ml.*	*per cent*
Hu	Zymosan*	130	100	75
"	Ammonia	100	< 50	< 50
G.p.	Zymosan	530	270	50
"	"	400	250	60
"	Ammonia	530	400	75
"	"	400	320	80
"	"	400	200	50
"	Hydrazine	400	200	50
"	"	530	200	40

* This was the only instance in which freshly boiled zymosan was used. C'2 recovery was 55 per cent when this pretreatment was omitted.

TABLE IV

Titration of C'3 in Hu and G.P. Complements

Human			Guinea pig		
Dilution for C'3 titration	Titer		Dilution for C'3 titration	Titer	
	Whole C'	C'3		Whole C'	C'3
1 to:	*units/ml.*	*units/ml.*	1 to:	*units/ml.*	*units/ml.*
50	80	350	60	500	600
25	100	200	50	400	350
25	80	350	40	400	300
25	100	350	40	400	400*
25	70	250	40	550	550*
25	100	>200	40	200	200
25	100	250	40	300	400
25	80	200	40	550	550
20	80	130	40	270	300
20	100	270	40	400	400
20	70	>130	40	300	300

* Same titer with fresh hu R3.

3. Titration of C'3.—R3, optimal for this titration, was prepared by treatment of g.p. C' with zymosan (2) according to (18), although the corresponding reagent from hu C' was also used in some instances. 0.05 ml. of 1:5 dilution (based on the g.p. C' used) appeared optimal for the titration of C'3 in hu C' and 0.1 ml. of the same dilution for the titration in g.p. C'. The former quantity of reagent contained roughly 10 units of C'1, 2 to 3 of C'2, and 20 of C'4. Results are recorded in Table IV from which it is evident that C'3 is the titer-limiting component of g.p. C', although the titers of C'2 are only slightly higher (Table II).

Titration for C'3 was carried out in M, E, and in ammonia-treated, and heat-inactivated sera.

Titration for C'3 in "Midpieces"

Source	Method of preparation	C'3 titer		Recovery
		Original C'	M	
		units/ml.	*units/ml.*	*per cent*
Hu	Dilution	200	50	25
"	"	200	30	15
"	Dialysis	200	100	50
"	" (pH 6)	200	100	50
"	"	200	30	15
G.p.	Dialysis, pH 5.4	270	100	35
"	" pH 6	270	100	35
"	"	400	170	40
"	Dilution	270	100	35

Titration for C'3 in Hu and G.P. "Endpieces"

Source	Method of preparation	C'3 titer		Recovery
		Original C'	E	
		units/ml.	*units/ml.*	*per cent*
G.p.	Dilution	270	60	20
"	Dialysis, pH 5.4	270	30	10
"	" pH 6.6	270	100	35
"	6 hrs. dialysis, pH 5.4	400	40	10
Hu	Dilution	200	30	15
"	"	200	60	30
"	Dialysis, pH 6.6	200	30	15
"	" pH 5.4	200	40	20

Titration for C'3 in Ammonia- or Hydrazine-Treated G.P. Sera

Source	C'3 titer		Recovery
	Original C'	NH_3-treated sera	
	units/ml.	*units/ml.*	*per cent*
G.p.	530	200	40
"	320	130	40
"	200	80	40
"	200	100*	50
"	270	130	50

* Serum treated with hydrazine.

Heat-inactivated g.p. C' also contains less C'3 than the original C', as shown below:—

C'3 titer		Recovery
Original C'	After 20 minutes at 56°C.	
units/ml.	*units/ml.*	*per cent*
400	270	70
320	200	60
270	130	50
320	250	80

The C'3 content of heated, NH_3-treated g.p. serum was also studied. After heating at 56°C. for 20 minutes, the samples were cooled in ice water and treated with ammonia in the usual way. This reagent should contain only C'3 in an active state:—

C'3 titer		Remaining C'3 activity
Original C'	C'3	
units/ml.	*units/ml.*	*per cent*
320	100	30
320	70	20
530	100	20

C'3 from human C' was also prepared, but its titer was only 20 units per ml. when tested with zymosan-treated hu C', and it was anticomplementary toward g.p. C'.

4. Titration of C'4.—R4 consisted preferably of ammonia-treated g.p. C' since the corresponding reagent from hu C' was anticomplementary (*cf.* also (2)) and contained too little C'2 and 3.

Usually 0.2 ml. of R4 at 1:10 dilution was used, the component content being 20 to 30 units of C'1, 6 of C'2, and 2 of C'3. The data are given in Table V.

TABLE V

Titration of C'4 in Hu and G.P. Complements

Human		Guinea pig	
Dilution	C'4 titer	Dilution	C'4 titer
1 to:	*units/ml.*	1 to:	*units/ml.*
300	4000*	400	5000*
500	5000*	400	5000
600	12,000*	300	3000
500	5000	300	6000
300	3000	300	3000
300	1200‡*	500	17,000
200	2000*	200	4000
500	2500*	500	7000
300	3000	500	10,000
200	2700	200	4000

* These values are possibly low, since in these instances optimal amounts of R4 were not necessarily used.

‡ In this case hu R4 was used.

C'4 titer of hu and g.p. "midpieces":—

Source	Method of preparation	C'4 titer		Recovery
		Original C'	M	
		units/ml.	*units/ml.*	*per cent*
Hu	Dilution	3000	130	4
"	Dialysis, pH 5.4	3000	200	10
"	" pH 6.6	3000	500	20
G.p.	" pH 5.4	2000	100	5
"	" pH 6.6	2000	100	5

Most of the C'4 of g.p. as well as hu C' is encountered in the "endpiece":—

Source	Method of preparation	C'4 titer		Recovery
		Original C'	E	
		units/ml.	*units/ml.*	*per cent*
Hu E	Dilution	3000	2400	80
" "	Dialysis, pH 5.4	3000	2400	80
" "	" pH 6.6	3000	2400	80
G.p. E	Dilution	4000	2000	50
" "	"	4000	3200	80
" "	6 hrs. dialysis, pH 5.4	6500	3000	45
" "	Dialysis, pH 5.4	10,000	4000	40

Treatment with zymosan (R3) also reduced the C'4 activity of hu and g.p. C':—

Source	C'4 titer*		Recovery
	Original C'	Reagent	
	units/ml.	*units/ml.*	*per cent*
Hu	5000	2000	40
G.p.	6000	2000	35
"	5000	2000	40

* Only g.p. R4 was used in these titrations. Titers were the same when 1 or 2 extra units of g.p. C'3 were added.

Although C'4 is considered a heat-stable component, an effect of heating sera at 56°C. for different periods was noted:—

Hu C'			G.p. C'		
Period heated	C'4 titer	Activity	Period heated	C'4 titer	Activity
min.	*units/ml.*	*per cent*	*min.*	*units/ml.*	*per cent*
0	3300	100	0	3300	100
5*	3000	90	5*	3000	90
10	1500	45	10	2000	60
15	800	25	15	1300	40
20	600	20	20	500	15
30	130	4	30	130	4

* Two minutes were added to each heating period for the sample to come to 56°C. after immersion in the bath.

Finally, reagents retaining only the reactivity of C'4 were prepared by treating heat-inactivated hu and g.p. C' with zymosan. With either of these reagents, g.p. R4 gave complete hemolysis. When the g.p. C'4 was added

to the somewhat anticomplementary ammonia- or hydrazine-treated hu C′ (hu R4) hemolysis did not occur. Replacement of hu C′4 by g.p. C′4 was easily accomplished (contrary to (2)), however, by addition of g.p. C′ or E, which contain much C′4, to hu R4.

Since these studies were carried out over a period of years and the methods adopted evolved gradually, complete data on all four components of every

TABLE VI

Titers of Hu and G.P. Complements and their Components

Source and No.	C′	C′1	C′2	C′3	C′4
	units/ml.	*units/ml.*	*units/ml.*	*units/ml.*	*units/ml.*
Individual, hu	70	2700	80	>130	3000
" "	100	5000	200	350	2500
" " 5	80	5000	130	350	2500
" " 7	100	4000	100	250	3000
Pool, " 4	100	2000	130	>200	2700
Pool, g.p.	300		800	350	6000
" "	500		500	600	17,000
" " 7	270	1000	270	300	7000
" " 8	300	1300	400	300	4000
" " 9	400	1600	400	400	10,000

TABLE VII

Average Titers of Complement and Components

Source	Whole C′	C′1	C′2	C′3	C′4
	units/ml.	*units/ml.*	*units/ml.*	*units/ml.*	*units/ml.*
G.p.	350	2300*	450	370	6000
Hu	100	3700*	170	250	4000

* Average titer with R1 (g.p. E plus heated g.p. serum), the optimal reagent.

complement were not obtained. Some of the more complete sets of titration values on individual complements are, however, taken from the various tables and given for ready comparison in Table VI. Numbers, when assigned, are those given in Table I.

The average titers found in the course of this study for hu and g.p. sera (Tables I, II, IV, and V) are given in Table VII.

DISCUSSION

Two principles, enunciated by Hegedüs and Greiner (1), have served as guides in the present study of the components of human and guinea pig complements: (1) the titer of a complement is limited by that of the component

present in lowest titer, and (2) each of the four components of complement may be titrated by making it the component of lowest titer through the addition of a reagent containing an excess of the other three components. The conditions in this way become analogous to those preferentially employed in the estimation of other biologically active substances. For example, comparisons of enzyme concentrations should be made at relatively high substrate concentrations so that the apparent enzyme values obtained are independent of substrate concentration (20). Similarly in studies of blood clotting, components other than the one to be measured should be present in excess so that the clotting time may vary only as a function of the amount of unknown component in the system.

The four reagents used in (1) for the study of the complements of a large number of animal species were prepared from guinea pig complement by standard methods: "endpiece" for the titration of C′1, "midpiece" for the titration of C′2, g.p. serum from which C′3 had been removed by the action of snake venom for the titration of C′3, and ammonia-treated g.p. serum, lacking in C′4, for the titration of C′4. No definite evidence was given, however, that the amounts of reagents used actually contained an excess of the desired components, or that they were always used below their anticomplementary concentrations.

In the present study each of the reagents used was subjected to scrutiny for its actual titer of the component or components it was expected to furnish, and its anticomplementary properties were checked in order to ascertain whether or not it might safely be used in concentrations high enough to supply the needed amounts.

It was soon found that "endpiece" (E), the standard reagent for C′1, was often too deficient in C′3 to provide more than a limiting titer, so that this remained the component of lowest titer in the mixture and was actually measured instead of C′1. It is believed that this accounts for the finding in (1) that the titer of C′1 in g.p. C′ is lower than that of C′2, whereas the present studies show the actual titer of C′1 to be very high. It was also noted that "midpiece" (M), the standard reagent for C′2, often failed to yield a measure of this component since it furnished too little C′3, in non-anticomplementary quantities, to raise the titer of this component in the test mixture above that of C′2. M was often deficient in its content of C′4, as well.

These defects proved to be readily remediable by addition of g.p. serum which had been heated at 56°C. for 20 minutes to all tests for C′1 and C′2. This not only provided an excess of C′3 (and C′4 if not already present), but also greatly reduced the often appreciable anticomplementary activity of M prepared from g.p. serum. Since human M was not anticomplementary and usually showed a considerably higher titer of C′1 than did g.p. M the reagent (R2) preferably used in these studies for the estimation of C′2

consisted of hu M + heated g.p. serum. The human E obtained as a by-product in the preparation of M is difficult to use owing to its low titer of C'2, so that for the titration of C'1, g.p. E + heated g.p. serum is recommended as the reagent (R1).

It was also found that in the so called "specific inactivation" (11, p. 424) of C'3 by "zymosan" (18, 2) and of C'4 by ammonia or hydrazine the other components of C' suffered more or less reduction as well. It is therefore proposed to abandon the inaccurate designation, "specifically inactivated" complements, and call these reagents R3 and R4, respectively, numbering them, as in R1 and R2, according to the components measured with their aid.

Although it is a simple matter to test a treated serum or reagent for anticomplementary action by its depression of the titer of whole complement, the evaluation of anticomplementary activity is not without complication. A treated serum or reagent may exert an enhancing action or an anticomplementary one, or it may have no effect at all on the titer of C'. Enhancing effects are attributable to the addition of the component, or components, present in lowest titer; *i.e.*, the titer-limiting component. Both enhancing and anticomplementary effects could, however, exist side by side in the same solution, but, of course, only the net effect could be demonstrated. In such an event the reagent might be anticomplementary toward g.p. C', for example, in which C'3 is usually the limiting component, but enhancing toward hu C', in which C'2 is the component in lowest titer. This would also explain the effect of heated g.p. C' in diminishing the anticomplementary action of g.p. M, since C'3 is added and this is the limiting component in g.p. C'. Possibly these circumstances also afford an explanation for the increase in titer of the C' by heated g.p. serum. While this would be expected to increase the titer of g.p. C', in which C'3 appears, on the average, to be the component of lowest titer (Tables II, IV, VI, and VII), although the titer of C'2 is not much higher, the reason for the increase is not so apparent in the case of hu C', in which C'2 is the limiting component. However, the titer of C'3 is not very much greater (Table VI). Since it is stated that C'3 is not fixed when C' is taken up in hemolysis (21, 13) it is possible that a considerable excess of this component is essential for full hemolytic activity, perhaps by driving back dissociation of a loose combination. Heated g.p. serum would provide such an excess. Other explanations are, however, possible, and the effect of heated serum requires further study.

Ecker and Pillemer and their coworkers have independently arrived at acceptance of underlying principles similar to those guiding the present studies,[3] so that necessary revisions of their published conclusions as to the components of complement may be awaited.

[3] Private communication from Dr. Ecker. *Cf.* also Ecker, E. E., Seifter, S., and Dozois, T. F., *J. Lab. and Clin. Med.*, 1945, **30**, 39.

Throughout the present paper emphasis has been laid on the titers of complement and its components. Since volume units have been used, and not units of weight, as, for example, in the quantitative method for whole complement (3–7), the data are purely relative and yield no information as to the actual content or concentration of any component. Nor do the titers even yield information as to the relative concentrations of the components, since equal quantities of each component are not necessarily required for hemolysis.

There appears, therefore, to be ample reason to adhere to the principles for the estimation of the components of complement laid down by Hegedüs and Greiner even though these authors did not themselves apply them consistently. While it is believed that this defect has been overcome in the present studies by the introduction of adequate controls, the writers are under no illusions as to the absolute values of the titers found, or as to each proposed reagent being optimally prepared or optimal for its purpose. They do believe, however, that building upon the work of Hegedüs and Greiner, they have placed the estimation of the components of complement upon a rational basis.

SUMMARY

1. Defects in methods previously proposed for the estimation of complement components are: failure to ensure an excess of the desired components and failure to ensure absence of anticomplementary effects in the dilution ranges used. Existing data are therefore subject to these uncertainties.

2. Methods are proposed for controlling the adequacy of the reagent for each component, for using it in dilutions below its anticomplementary range, and for reinforcing it with necessary components if these are present in inadequate amounts.

3. Titrations are given of the four components in human and guinea pig complements and in "midpiece," "endpiece," and in the various reagents used, including one with C′3 reactivity, solely, and one with C′4 reactivity.

4. C′3 is shown to be the component which usually limits the titer in guinea pig complement, and C′2 the component of lowest titer in human complement.

5. In immune hemolysis, each component of human complement may be replaced by the corresponding component of guinea pig complement and *vice versa*.

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