FLUID PHASE DESTRUCTION OF $C2^{hu}$ BY $C1^{hu}$

I. Its Enhancement and Inhibition by Homologous and Heterologous C4*

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Lepow and associates reported in 1954 (1) that the activation of the first component of complement (C1)[1] was associated with the capacity to inactivate the fourth (C4) and to a lesser degree the second (C2) components of human complement in the pseudoglobulin fraction of serum. These results were later confirmed with partially purified preparations of human C1 (2), with eluates of immune aggregates pretreated with fresh human serum (3), and with a highly purified C1s fraction of the C1 macromolecule (4). More recently, Müller-Eberhard and Lepow (5) have described physicochemical changes in isolated C4 following interaction with highly purified C1s.

The classical studies of Mayer and coworkers (6) clearly demonstrated that cell-bound first component, EAC1, could not uncover the binding site of C2 unless C4 was already fixed to the cell, EAC14. Subsequently, Becker (7) established that the esteratic activity of C1 must be intact for the EAC14 intermediate to interact with C2 so as to achieve the EAC142 state. This was confirmed by Stroud, Austen, and Mayer (8) with the demonstration that the interaction of EAC14 intermediate with C2 was associated with the appearance of an altered, hemolytically inactive form of C2 in the fluid phase. Neither the altered form of C2 nor the EAC142 intermediate developed if the enzymatic activity of the EAC14 complex was inactivated by pretreatment with diisopropyl fluorophosphate (DFP).

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[1] The nomenclature used conforms to that agreed upon at the Fifth Complement Workshop held in Boston, Massachusetts, in June, 1968. Sheep erythrocytes (E), sensitized with rabbit antibody (A), react with the components of complement (C) in the sequence, C1,4,2,3, 5,6,7,8, and 9. Fragments of individual components are subscripted with letters. The activated forms of the components are assumed to exist in intermediate cellular complexes unless otherwise specified. In addition to these conventions, the species of origin of a given component is indicated by a superscript (hu, human; gp, guinea pig). SAC14 and SAC142 refer to the proportion of hemolytically active sites formed per erythrocyte during the interaction of EAC1 with C4 and EAC14 with C2, respectively.

Müller-Eberhard (9, 10) has established that native C4 and C2 isolated from human serum can interact reversibly under defined conditions, and that the product of the action of C1s on C4 and C2 is a new enzyme, consisting of inactivated C4 (C4i) and a fragment of C2 (C2a) termed C3 convertase. Although it is clear that C4 is a natural substrate of C1 and their interaction must precede the participation of C2 in the hemolytic sequence, the nature of the process by which C4 facilitates the interaction of C1 with C2 is not established.

The present studies examine the interaction of C1 and C2 in the fluid phase and demonstrate that homologous C4 enhances the inactivation of C2 by C1, whereas heterologous C4 is inhibitory. Thus, in the fluid phase as on the cellular intermediate, C4i is essential to the specific action of C1 on C2.

Materials and Methods

The sources and methods of handling of sheep erythrocytes, guinea pig serum, and human sera have been described (11). The methods for preparing Veronal-buffered saline, pH 7.5, 0.145 M, containing 0.1% gelatin, 0.00015 M Ca^{++} and 0.0005 M Mg^{++} (GVB^{++}), and dextrose-Veronal–buffered saline, 0.075 M, with the same concentrations of gelatin and cations ($D\text{-}GVB^{++}$) have been reported (12). Disodium ethylenediaminetetraacetate (EDTA) buffers in GVB were prepared to a final concentration of 0.01 M and 0.04 M EDTA (13). The sources and method of preparation of diethylaminoethyl (DEAE)- and carboxymethyl (CM)-celluloses, and the buffers used with them, have been previously described (13). Diisopropyl fluorophosphate (DFP), molecular weight 184.15, reagent grade (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was used as described by Becker (7).

Preparation of Human and Guinea Pig Components of Complement.—

Human and guinea pig first component: $C1^{hu}$ and $C1^{gp}$ were precipitated at pH 7.5, with a conductivity equivalent to 0.04 M NaCl, as described by Nelson et al. (13) and Vroon and Nelson.[2] The precipitate formed was washed in saline phosphate buffer at the same pH and ionic strength, redissolved in 0.3 M NaCl in 0.005 M phosphate buffer, pH 7.5, and reprecipitated and redissolved again using the same buffers. Further purification was obtained by filtration through Sephadex G-200 equilibrated with 0.15 M NaCl in 0.005 M phosphate buffer, pH 7.5 (14). The C1 activity was contained in an elution volume equivalent to the first protein peak obtained when whole serum was applied to the column.

Human and guinea pig fourth component: The techniques of Vroon and Nelson[2] and Nelson et al. (13) were used. After precipitation of the euglobulins from 100 ml of human serum or guinea pig serum by procedures identical with those described above for C1, the supernatant was adjusted to ionic strength 0.120 M NaCl and applied to a DEAE-cellulose column (5.0 × 50 cm) equilibrated at pH 7.5, 0.120 M NaCl. When the application of the protein was completed, the column was washed with starting buffer until the optical density at 280 mμ of the effluent was less than 0.050. A straight line gradient of increasing salt concentration was then applied. C4 eluted at 0.140–0.150 M NaCl but was heavily contaminated with the inhibitor of C1 esterase. Fractions containing C4 activity were pooled, adjusted to 0.090 M NaCl by dilution with distilled water and to pH 5.0 by the addition of 1.0 M acetic acid, and applied to a CM-cellulose column equilibrated at the same pH and ionic strength. The C1 esterase inhibitor passed through the CM-cellulose column in the effluent, and C4 was eluted with a linear salt gradient at 0.120–0.130 M NaCl. The C4 preparations obtained by

[2] Vroon, D., and R. A. Nelson. Unpublished data presented at the Complement Workshop, La Jolla, California, 1966.

this technique ranged from 1×10^{11} to 5×10^{11} effective molecules/ml and were free of functional activity of other components. Yields ranged from 10 to 25% of the $C4^{hu}$ or $C4^{gp}$ measured in the original serum.

Human second component: The initial 0.120 M NaCl effluent from the DEAE column used for the preparation of $C4^{hu}$ contained $C2^{hu}$ activity as well as the activities of the other complement components of the serum with the exception of C1, C4, and C9. The effluent fractions were pooled and brought to a final concentration of 2.2 M $(NH_4)_2SO_4$ by the addition of 4.0 M $(NH_4)_2SO_4$; this precipitated most of the complement components other than $C2^{hu}$ and $C8^{hu}$. The supernatant was dialyzed against several changes of phosphate buffer, 0.075 M NaCl, pH 7.5, adjusted to 0.080 M NaCl, pH 6.0, by the addition of 1.0 M acetic acid and applied to a CM-cellulose column which had previously been equilibrated at the same pH and ionic strength. After washing the column with starting buffer, a linear salt gradient was applied and $C2^{hu}$ eluted at 0.120–0.130 M NaCl.[2] This procedure produced functionally pure $C2^{hu}$ with activities ranging from 2×10^{10} to 6×10^{10} effective molecules/ml, with yields of approximately 10% of the $C2^{hu}$ activity of the original serum.

Guinea pig second component: The supernatant fluid from the precipitation of C1 was adjusted to 0.075 M NaCl and passed through a DEAE-cellulose column equilibrated at the same ionic strength and pH 7.5. All the $C2^{gp}$ activity was found in the initial effluent, which also contained C6 and some C7 activity. Fractions containing C2 activity were pooled, adjusted to pH 5.0, 0.075 M NaCl, and applied to a CM-cellulose column equilibrated at the same pH and ionic strength. The $C2^{gp}$ passed through this column with the effluent, and the C6 and C7 were retained. The final C2 preparation contained trace amounts of C7 as the only functional contaminant when tested by the techniques described by Nelson et al. (13). The activity was 7.5×10^{10} effective molecules/ml and represented approximately 40% of the $C2^{gp}$ activity of the original serum.

Human C1 inhibitor (C1 INH): The techniques described by Gigli et al. (15) were used to isolate the C1 INH from human serum.

Cellular Intermediates of the Hemolytic System and Titration of Components of Complement.— Sheep erythrocytes were coated with commercial anti-sheep hemolysin made in rabbits or with purified rabbit 19S antibody (EA) isolated from rabbits immunized with sheep E stromata. $EAC1^{gp}$ cells were prepared by mixing EA in $D\text{-}GVB^{++}$ with an equal volume of partially purified $C1^{gp}$ diluted to provide 250 effective molecules/cell in the fluid phase; after incubation at 30°C for 45 min, the cellular intermediate was washed three times in $D\text{-}GVB^{++}$ and stored in the same buffer with penicillin and streptomycin (16). $EAC1^{gp}4^{hu}$ were prepared from the $EAC1^{gp}$ cells either by the method described by Borsos and Rapp (17) or by incubating, at 30°C for 30 min, $EAC1^{gp}$ with purified $C4^{hu}$ diluted in $D\text{-}GVB^{++}$ to provide 200 effective C4 molecules/cell in the fluid phase. $EAC4^{hu}$ cells were obtained by incubating $EAC1^{gp}4^{hu}$ in 0.01 EDTA-GVB according to the method of Borsos and Cooper (18). All cellular intermediates were washed and stored as described for $EAC1^{gp}$. The procedures for the effective molecule titrations of C1 (19), C4 (16), and C2 (20), human or guinea pig, have been previously described.

RESULTS

The Fluid Phase Destruction of $C4^{hu}$ by Homologous and Heterologous C1.— 0.5 ml samples of $C4^{hu}$, 4 units/ml,[3] were mixed with 0.5 ml of $D\text{-}GVB^{++}$ or 0.5 ml of a serial dilution of $C1^{hu}$ or $C1^{gp}$ at concentrations ranging from 0.375

[3] Concentration of components in the fluid phase is expressed as units/ml. One unit is the dilution sufficient to generate one hemolytically active site per cell at a concentration of 1×10^8 cells/ml.

to 6.0 C1 units/ml. After incubation for 30 min at 30°C, 0.5 ml of $EAC1^{gp}$ at 1×10^8 cells/ml was added to each tube and the reaction mixtures incubated for 15 min at 30°C. 0.5 ml of D-GVB^{++} containing 100 effective molecules of $C2^{gp}$ was then added and the reaction mixtures incubated for an additional 15 min at 30°C. 0.5 ml of 0.04 M EDTA-GVB followed by 1.0 ml of a 1:15 dilution of guinea pig complement (GPC) in 0.04 M EDTA were added to each tube and

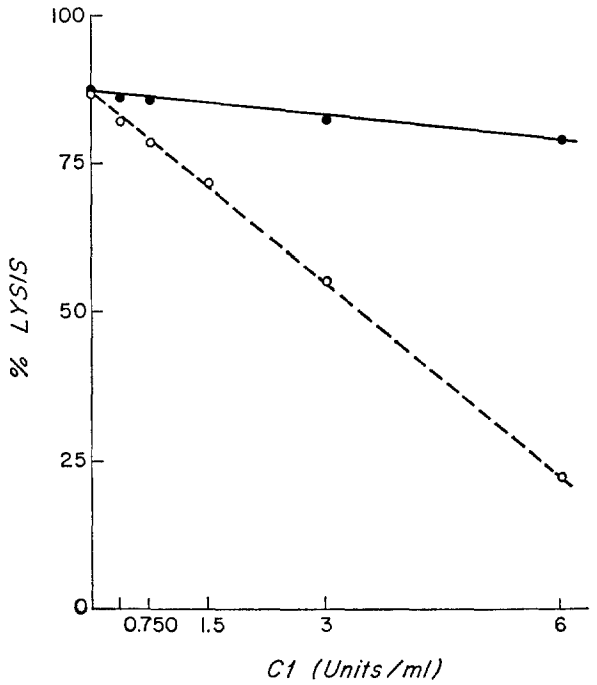

FIG. 1. Fluid phase destruction of $C4^{hu}$ by varying amounts of $C1^{hu}$ (○) and $C1^{gp}$ (●). The residual $C4^{hu}$ was measured by the conversion of $EAC1^{gp}$ to $EAC1^{gp}4^{hu}$.

the samples incubated for 60 min at 37°C. After the addition of 4.0 ml of ice cold saline, the per cent lysis was determined (16). Fig. 1 shows that the conversion of $EAC1^{gp}$ to $EAC1^{gp}4^{hu}$ by the residual $C4^{hu}$ decreased as a linear function of the $C1^{hu}$ present in the original mixture of C1 and C4. When $C1^{gp}$ was present in the mixtures, the destruction of $C4^{hu}$ was only 12% of that produced by the same molecular concentration of $C1^{hu}$.

The kinetics of fluid phase destruction of $C4^{hu}$ by $C1^{hu}$ and the possible changes in hemolytic activity of the $C1^{hu}$ during this reaction were studied in the following experiment. Three 5 ml samples of $C4^{hu}$, 4 units/ml, were preincubated at 30°C. At time zero, the samples received an equal volume of D-

GVB^{++}, $C1^{hu}$, 0.8 units/ml, or $C1^{hu}$, 0.6 units/ml, respectively. Immediately after mixing and at 5 min intervals thereafter, 0.5 ml samples were removed from each reaction mixture and added to 0.5 ml of $EAC1^{gp}$ for a titration of the residual hemolytically active $C4^{hu}$ as described above. Duplicate 0.5 ml samples were removed at the same intervals and added to 0.5 ml of $EAC4^{hu}$ for titration of the residual hemolytically active $C1^{hu}$. These reaction mixtures were incubated for 30 min at 30°C, followed by the addition of 0.5 ml $C2^{gp}$ containing 100 effective molecules. After 15 min of incubation at 30°C, 0.5 ml of 0.04 M EDTA, followed by 1.0 ml of a 1:15 dilution of GPC in 0.04 M EDTA were added, and

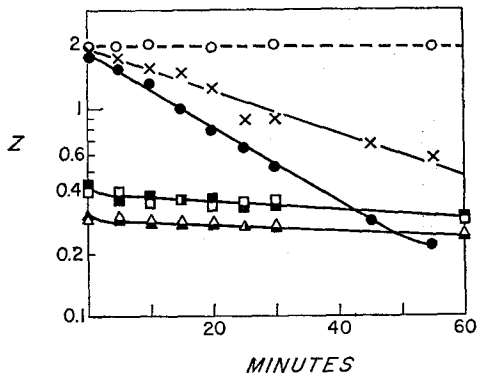

FIG. 2. Kinetics of fluid phase destruction of $C4^{hu}$ by 0.6 units $C1^{hu}$ (×) or 0.8 units $C1^{hu}$ (●). The concentration of $C4^{hu}$ in buffer alone is indicated (○). The concentration of $C1^{hu}$, 0.6 units/ml (▲ △) and 0.8 units/ml (■ □) in buffer and in the experimental tubes containing $C4^{hu}$, respectively, is also indicated. Z refers to the proportional number of hemolytically active sites formed per erythrocyte.

the samples further incubated for 60 min at 37°C. After the addition of 4.5 ml ice cold saline, the per cent lysis was determined (19). Control samples taken from two additional mixtures of the same starting $C1^{hu}$ concentrations in D-GVB^{++} were analyzed for $C1^{hu}$ in the same manner. Fig. 2 shows the residual $C4^{hu}$ and $C1^{hu}$ in each reaction mixture as measured by the formation of SAC14. With both $C1^{hu}$ concentrations there was a linear decrease in $C4^{hu}$ activity during the 60 min incubation. The $C1^{hu}$ activity in the C1-C4 reaction mixtures was not reduced compared with $C1^{hu}$ incubated with buffer alone.

The Fluid Phase Destruction of $C2^{hu}$ or $C2^{gp}$ by Homologous and Heterologous C1.—1 ml samples of $C2^{hu}$, 4 units/ml, were mixed with 1 ml of D-GVB^{++} or serial dilutions of $C1^{hu}$ or $C1^{gp}$ ranging from 106.25 to 1700 C1 units/ml. An identical series of reaction mixtures was prepared using $C2^{gp}$ instead of $C2^{hu}$. After incubation for 30 min at 30°C, a 0.5 ml aliquot was removed from each reaction mixture and added to 0.5 ml of $EAC1^{gp}4^{hu}$. After incubation for 3 min

at 30°C, 1.5 ml of a 1:22.5 dilution of GPC in 0.04 M EDTA was added; the mixtures were incubated for 90 min at 37°C and the per cent lysis determined (20). Fig. 3 shows that the conversion of $EAC1^{gp}4^{hu}$ to $EAC1^{gp}4^{hu}2^{hu}$ or $EAC1^{gp}4^{hu}2^{gp}$ by the residual $C2^{hu}$ or $C2^{gp}$ in each reaction mixture decreased as a linear function of the $C1^{hu}$ or $C1^{gp}$ concentration. When $C1^{gp}$ was present in the mixtures, the destruction of $C2^{hu}$ was only 12% of that produced by the

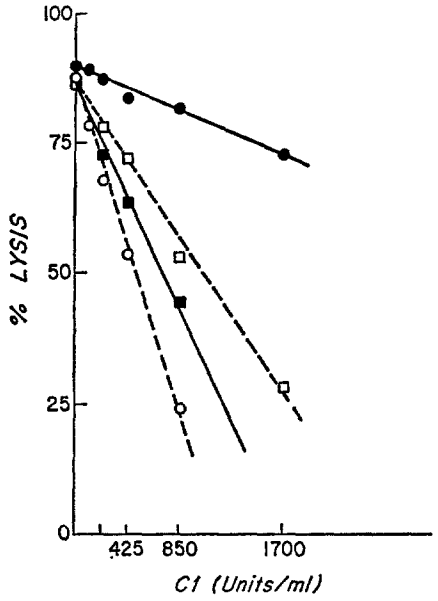

FIG. 3. Fluid phase destruction of $C2^{hu}$ or $C2^{gp}$ by varying amounts of $C1^{hu}$ or $C1^{gp}$. Reaction of $C2^{hu}$ with $C1^{hu}$ (○) or $C1^{gp}$ (●), and of $C2^{gp}$ with $C1^{hu}$ (□) or $C1^{gp}$ (■), were assessed by determining residual C2 by the conversion of $EAC1^{gp}4^{hu}$ to $EAC1^{gp}4^{hu}2^{hu}$ or $EAC1^{gp}4^{hu}2^{gp}$.

same molecular concentration of $C1^{hu}$. The concentrations of C1 selected were based on the preliminary finding that the destruction of C2 required more than 50 C1 units/ml.

The kinetics of fluid phase destruction of $C2^{hu}$ by $C1^{hu}$ were studied with a wide range of $C1^{hu}$ concentrations. Four 5 ml samples of $C2^{hu}$, 15 units/ml, were preincubated at 30°C and then reacted with either 5 ml of D-GVB^{++} or 5 ml of $C1^{hu}$ containing 50, 500, or 5000 units/ml. Immediately after mixing and at 5 min intervals thereafter, 0.5 ml samples were removed and diluted to 5 ml with D-GVB^{++}; 0.5 ml of each dilution was mixed with 0.5 ml of $EAC1^{gp}4^{hu}$ to determine the residual hemolytically active $C2^{hu}$, as described above. Fig. 4 shows the $C2^{hu}$ hemolytic activity in the four reaction mixtures expressed as $SAC1^{gp}4^{hu}2^{hu}$ formed. The $C2^{hu}$ activity in the samples incubated with buffer

only remained constant throughout the 60 min of incubation. When $C1^{hu}$ containing 50 units/ml was added, the loss of hemolytic activity reached only 12% of the total $C2^{hu}$ present. At a $C1^{hu}$ concentration of 500 units/ml, the $C2^{hu}$ destruction proceeded at a faster rate; and in 60 min 90% of the C2 was no longer detectable. The same decrease in C2 activity was achieved in 7 min when the concentration of $C1^{hu}$ was increased to 5000 units/ml. In the three reaction mixtures, there was a lag phase of 3 min during which C2 was stable regardless of the $C1^{hu}$ concentration. The stability of $C1^{hu}$ during this reaction was studied in a separate experiment where 125 units of $C1^{hu}$ were incubated either with 10 units of $C2^{hu}$ or with D-GVB^{++}. The $C1^{hu}$ activity was identical whether it was incubated with $C2^{hu}$ or with buffer only.

The Fluid Phase Destruction of $C2^{hu}$ by $C1^{hu}$ in the Presence of $C4^{hu}$.—Three 5 ml aliquots of $C2^{hu}$, 3 units/ml, were incubated at 30°C with 5 ml of D-GVB^{++}, 2.5 ml D-GVB^{++}, and 2.5 ml $C1^{hu}$ containing 500 units/ml, or 2.5 ml of the

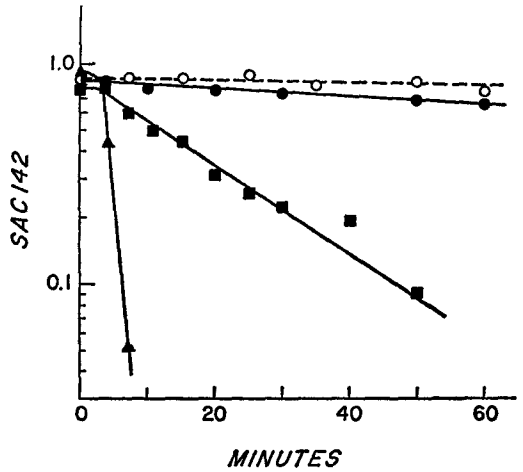

FIG. 4. Kinetics of fluid phase destruction of $C2^{hu}$ by 50 (●), 500 (■), or 5000 (▲) units $C1^{hu}$/ml, respectively. O refers to the $C2^{hu}$ incubated with buffer alone.

same $C1^{hu}$ and 2.5 ml of $C4^{hu}$ containing 50 units/ml. Immediately and at 7.5 min intervals, 0.5 ml aliquots were removed and added to 0.5 ml of $EAC1^{sp}4^{hu}$ for a titration of the residual functional $C2^{hu}$. The results shown in Fig. 5 reveal that in the presence of $C4^{hu}$ one-half of the functional $C2^{hu}$ is destroyed within 30 sec. Thereafter, the disappearance of hemolytically active C2 proceeds at a rate comparable to its loss in the mixture of $C2^{hu}$ and $C1^{hu}$ alone.

To investigate the effect of the $C4^{hu}$ concentration on the fluid phase reaction of $C1^{hu}$ and $C2^{hu}$, an experiment was performed in which a constant amount of $C1^{hu}$ and a limited amount of $C2^{hu}$ were interacted in the presence of varying dilutions of $C4^{hu}$. Six 5 ml samples of $C2^{hu}$, 1.5 units/ml, were incubated with 5 ml D-GVB^{++}, 2.5 ml D-GVB^{++}, and 2.5 ml $C1^{hu}$ containing 100 units/ml, or with 2.5 ml of four different concentrations of $C4^{hu}$ containing from 9.2 to 250

units/ml and 2.5 ml of the same $C1^{hu}$ (100 units/ml). All the dilutions were prewarmed at 30°C and mixed in the order described. Immediately and at varying time intervals, as designated in Fig. 6, 0.5 ml samples were removed and added to 0.5 ml of $EAC1^{sp}4^{hu}$ for a titration of hemolytically active $C2^{hu}$. The results reveal that increasing concentrations of $C4^{hu}$ in the reaction mixtures are associated with an increasingly rapid initial destruction of $C2^{hu}$ by $C1^{hu}$. The finding that the action of $C1^{hu}$ on $C2^{hu}$ in the absence of additional $C4^{hu}$ also had an initial, more rapid, slope may indicate contamination of this $C1^{hu}$ preparation with $C4^{hu}$ not detected by functional titration. $C2^{hu}$ incubated with buffer remained constant throughout the experiment. Additional control

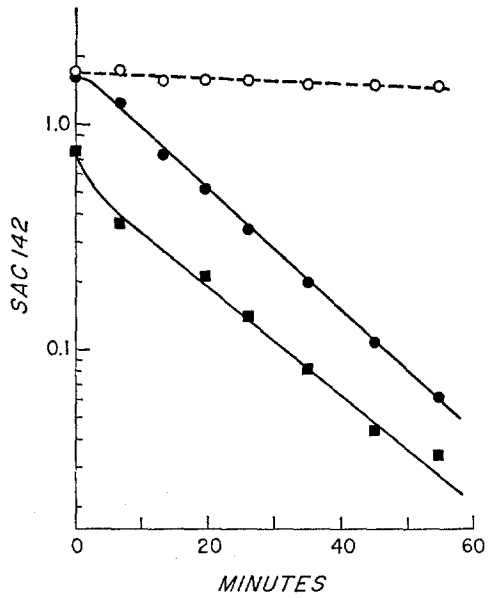

FIG. 5. The fluid phase destruction of $C2^{hu}$ by $C1^{hu}$ alone (●) and in the presence of $C4^{hu}$ (■). ○ refers to the $C2^{hu}$ incubated with buffer alone.

mixtures of $C4^{hu}$ and $C2^{hu}$ without C1 revealed that the amounts of $C4^{hu}$ used in this experiment had no effect on the activity of C2 compared to the $C2^{hu}$ sample incubated with buffer.

The Fluid Phase Destruction of $C2^{hu}$ by $C1^{hu}$ in the Presence of C4 Previously Inactivated by Exposure to $C1^{hu}$.—The observation that the destruction of $C2^{hu}$ by $C1^{hu}$ in the presence of $C4^{hu}$ had an initial rapid and subsequent slow phase introduced the possibility that the rapid phase was dependent upon the presence of hemolytically active C4. An experiment was performed to determine the effect of hemolytically active and inactive C4 on C2 destruction by C1. $C4^{hu}$, 50 units/ml, was incubated at 30°C with an equal volume of $C1^{hu}$, 100 units/ml.

Immediately upon mixing and at varying times thereafter, duplicate 0.5 ml samples were removed. One sample was diluted 10-fold in D-GVB^{++}, and 0.5 ml of this dilution was added to 0.5 ml of EAC1 cells to measure the residual hemolytically active C4. The second sample was added to 0.5 ml of $C2^{hu}$, 2 units/ml, and the mixture incubated for 30 min at 30°C; 0.5 ml of $EAC1^{sp}4^{hu}$

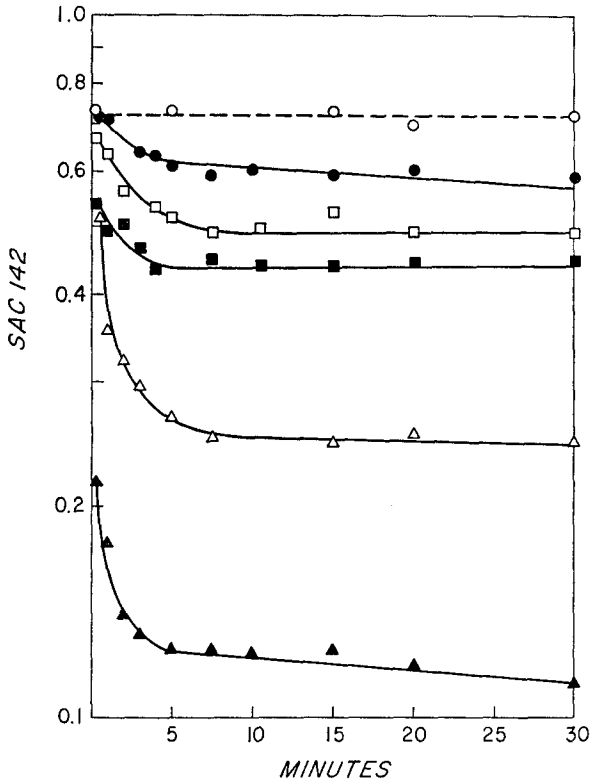

FIG. 6. The fluid phase destruction of $C2^{hu}$ by $C1^{hu}$ alone (●), and in the presence of 4.6 (□), 13.2 (■), 41.6 (△), and 125 (▲) $C4^{hu}$ units. ○ refers to the $C2^{hu}$ incubated with buffer alone.

was then added to determine the residual hemolytically active $C2^{hu}$. Two control samples of $C2^{hu}$ received either 0.5 ml D-GVB^{++} or 0.5 ml of $C1^{hu}$ at a concentration equal to that in the $C1^{hu}$-$C4^{hu}$ mixture. Fig. 7 shows that $C2^{hu}$ incubated with buffer alone generated 2.0 SAC142, $C2^{hu}$ incubated with $C1^{hu}$ yielded 1.55 SAC142, while $C2^{hu}$ incubated with the $C1^{hu}$-$C4^{hu}$ mixture yielded only 0.65 SAC142 at a time when the C4 was hemolytically inactive. The residual $C2^{hu}$ exposed to the $C1^{hu}$-$C4^{hu}$ mixture at zero time, when $C4^{hu}$ was fully hemolytically active yielded 0.82 SAC142. Thus, the $C1^{hu}$-$C4^{hu}$ mixture was

capable of destroying $C2^{hu}$ in the fluid phase at a time when the $C4^{hu}$ was inactivated (C4i) by prior exposure to $C1^{hu}$.

The Inability to Achieve Fluid Phase Destruction of $C2^{hu}$ in the Presence of C4i and Blocked $C1^{hu}$.—Because of the possibility that the $C2^{hu}$ depletion was the result of an association with C4i rather than a consequence of C1 action in the

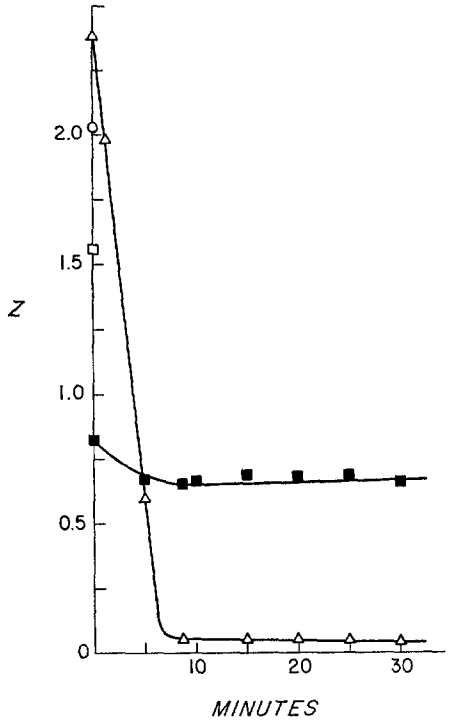

FIG. 7. The fluid phase destruction of $C2^{hu}$ by $C1^{hu}$ alone (□) and by $C1^{hu}$–$C4^{hu}$ mixtures (■) previously interacted for varying time intervals. △ refers to the residual hemolytically active $C4^{hu}$ in the $C1^{hu}$–$C4^{hu}$ mixtures. ○ refers to the $C2^{hu}$ incubated with buffer alone.

presence of C4i, it was necessary to examine the effect of C4i on C2 activity. C4i was produced by the interaction of 1 ml $C4^{hu}$, 50,000 units/ml, and 1 ml of $C1^{hu}$, 60,000 units/ml, for 30 min at 30°C. A second sample of 1 ml C4, 50,000 units/ml, was incubated with 1 ml D-GVB^{++}. 1 ml of the C4i and 1 ml of the control C4 received DFP to a final concentration of 0.005 M/ml. All four samples, C4, C4 treated with DFP, C4i-$C1^{hu}$, and C4i-$C1^{hu}$ treated with DFP, were incubated for 10 min at 30°C, followed by dialysis against three 1000 ml D-GVB^{++} changes. 0.5 ml of serial dilutions of each of the four mixtures was then mixed with 0.5 ml of $C2^{hu}$, 2.2 units/ml. After 30 min of incubation at 30°C, 0.5

ml of EAC14 cells was added to determine the residual hemolytically active $C2^{hu}$. As shown in Fig. 8, the C2 incubated with D-GVB^{++} alone yielded 2.1 SAC142. The C2 incubated with the 1:300 dilutions of C4, C4 pretreated with DFP, or C4i-$C1^{hu}$ pretreated with DFP yielded 2.1, 1.93, and 1.8 SAC142, respectively; whereas C2 incubated with C4i-$C1^{hu}$ yielded only 0.73 SAC142. The $C1^{hu}$ activity in the C4i-C1 mixture was the same as that found in the sample of $C1^{hu}$ added to form the mixture, whereas the C4i-$C1^{hu}$ treated with

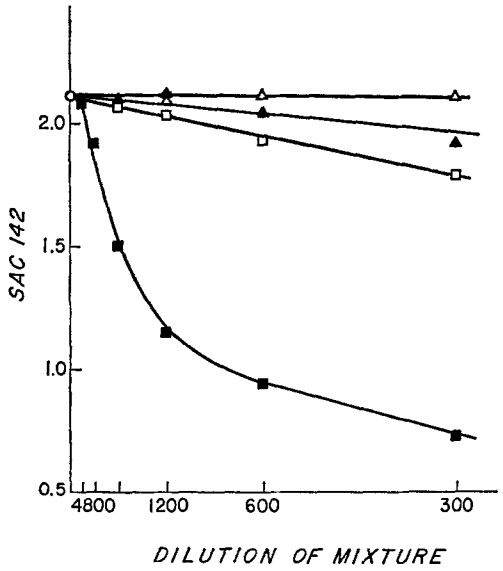

FIG. 8. Fluid phase destruction of $C2^{hu}$ by $C1^{hu}$–C4i^{hu} mixtures before (■) and after (□) DFP treatment. ○ refers to $C2^{hu}$ incubated with buffer alone, △ to $C2^{hu}$ incubated with $C4^{hu}$, and ▲ to $C2^{hu}$ incubated with $C4^{hu}$ DFP-treated.

DFP had lost 98% of the $C1^{hu}$ activity. The C4i-C1 mixtures with or without DFP treatment had no demonstrable C4 hemolytic activity, while the activity in the C4 samples with and without DFP treatment was the same as that added at the start of the experiment. These data reveal that the destruction of $C2^{hu}$ facilitated by C4i requires enzymatically active C1.

This point was examined further by employing C1 INH in place of DFP to inhibit C1. Three 1 ml samples of $C1^{hu}$, 222 units/ml, were incubated for 30 min at 30°C with an equal volume of two dilutions of C1 INH containing 300 and 600 units/ml respectively, or with D-GVB^{++}. 1 ml of $C4^{hu}$, 160 units/ml, and 1 ml of $C2^{hu}$, 45 units/ml, were then added. A control sample of $C1^{hu}$ and a control sample of $C4^{hu}$-$C2^{hu}$ mixture were prepared to give the same concentration as was contained in the experimental tubes. After 30 min at 30°C, dilutions of

the five reaction mixtures were titrated for $C1^{hu}$, $C4^{hu}$, and $C2^{hu}$ hemolytic activity. The results in Table I reveal that the concentration of inhibitor selected, 300 and 600 units/ml, permitted the reduction of $C4^{hu}$ to 0 and 15.5 units/ml, respectively, from a starting level of 160 units/ml, even though the $C1^{hu}$ activity had been reduced from 222 to 7.2 and 2.8 units/ml, respectively. On the other hand, the C2 activity which was reduced from 45 to 4 units/ml by the presence of C1 and C4 was protected almost completely by both concentrations of inhibitor.

The Fluid Phase Destruction of $C2^{hu}$ by $C1^{hu}$ in the Presence of Homologous and Heterologous C4.—The species specificity of the enhancement effect of C4 upon $C2^{hu}$ destruction by $C1^{hu}$ was studied by comparing the effect of $C4^{gp}$ with $C4^{hu}$. Four 2 ml samples of $C2^{hu}$, 6 units/ml, were mixed with 4 ml D-GVB^{++};

TABLE I

The Effect of C1 INH on the Inactivation of $C4^{hu}$ and $C2^{hu}$ by $C1^{hu}$

Reaction mixture	C1	C2	C4
	units/ml		
C1 + D-GVB^{++}	222		
C4 + C2		45	160
C1 + C4 + C2	236	4	0
C1 + C1 INH 300 units + C4 + C2	7.2	40	0
C1 + C1 INH 600 units + C4 + C2	2.8	42	15.5

2 ml D-GVB^{++} and 2 ml of C1, 60 units/ml; 2 ml $C4^{hu}$, 100 units/ml, and 2 ml of the same $C1^{hu}$; or 2 ml $C4^{gp}$, 100 units/ml, and 2 ml $C1^{hu}$. The four mixtures were incubated at 30°C. Immediately and at varying time intervals, 0.5 ml samples were removed and added to 0.5 ml of $EAC1^{gp}4^{hu}$ to determine residual hemolytically active C2. As shown in Fig. 9, after 30 min of incubation the $C2^{hu}$ in buffer yielded 1.95 SAC142; that exposed to $C1^{hu}$, 1.25 SAC142; and that exposed to $C1^{hu}$ in the presence of homologous C4, 0.58 SAC142. When $C2^{hu}$ was exposed to $C1^{hu}$ in the presence of heterologous C4, there was no loss of $C2^{hu}$ in that 2.0 SAC142 were formed. $C1^{hu}$ titrations of the three mixtures containing this component revealed no loss of its activity, and thus the protection afforded by heterologous C4 cannot be attributed to the presence of C1 INH in this preparation.

The finding that C2 destruction by C1 was enhanced by homologous C4 and prevented by heterologous C4 prompted studies on the effect of mixtures of homologous and heterologous C4 on the $C2^{hu}$ destruction by $C1^{hu}$. Five 1 ml samples of C2, 2.6 units/ml, were incubated at 30°C. Two of these samples received 1 ml $C4^{hu}$, 100 units/ml; two received 1 ml $C4^{gp}$, 100 units/ml; and one received 2 ml D-GVB^{++}. After a 10 min incubation, 1 ml of either $C4^{gp}$, 100

units/ml, or 1 ml D-GVB^{++} was added to the samples containing $C2^{hu}$-$C4^{hu}$; and 1 ml $C4^{hu}$, 100 units/ml, or 1 ml D-GVB^{++} to the mixtures of $C2^{hu}$-$C4^{gp}$. After a 10 min incubation at 30°C, 1 ml $C1^{hu}$, 200 units/ml, was added to the five reaction mixtures. A control sample containing 1 ml C2, 3 units/ml, was mixed with 3 ml D-GVB^{++}. After 30 min at 30°C, 0.5 ml was removed from the six samples and added to 0.5 ml of $EAC1^{gp}4^{hu}$ to determine the residual hemolytically active $C2^{hu}$. Fig. 10 shows that the C2 incubated with buffer yielded 0.87 SAC142 (A); that exposed to $C1^{hu}$, 0.39 SAC142 (E); and that exposed

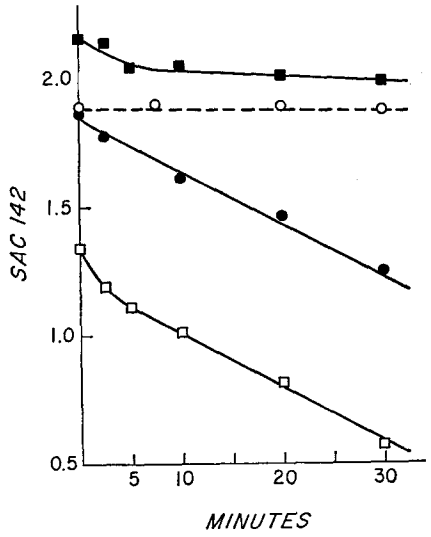

FIG. 9. The fluid phase destruction of $C2^{hu}$ by $C1^{hu}$ (●) and in the presence of homologous (□) and heterologous (■) C4. ○ refers to $C2^{hu}$ incubated with buffer alone.

to $C1^{hu}$ in the presence of homologous C4, 0.19 SAC142 (F). The substitution of heterologous for homologous C4 generated 0.92 SAC142 (B). When homologous and heterologous C4 were present in equal concentrations, regardless of the order of their addition, there was partial protection in that 0.56 SAC142 (C, D) was generated.

The competition of homologous and heterologous C4 was also examined in a kinetic experiment. Five samples of 1 ml $C1^{hu}$, 100 units/ml, were incubated at 30°C. Two of these samples received 1 ml $C4^{hu}$, 200 units/ml; two received 1 ml $C4^{gp}$, 200 units/ml; and one was mixed with 2 ml D-GVB^{++}. After 10 min of incubation at 30°C, 1 ml of either $C4^{gp}$, 200 units/ml, or D-GVB^{++} was added to the samples containing $C1^{hu}$-$C4^{hu}$; and 1 ml $C4^{hu}$, 200 units/ml, or 1 ml D-GVB^{++} was added to the samples containing $C1^{hu}$-$C4^{gp}$. All five experimental

mixtures received 1 ml $C2^{hu}$, 4 units/ml. Control samples included $C2^{hu}$ alone, $C2^{hu}$ mixed with $C4^{gp}$, and $C2^{hu}$ mixed with $C4^{hu}$. At zero time and at varying time intervals, 0.5 ml samples were removed and added to 0.5 ml of $EAC1^{gp}4^{hu}$ to titrate the residual hemolytically active $C2^{hu}$. As shown in Fig. 11, the re-

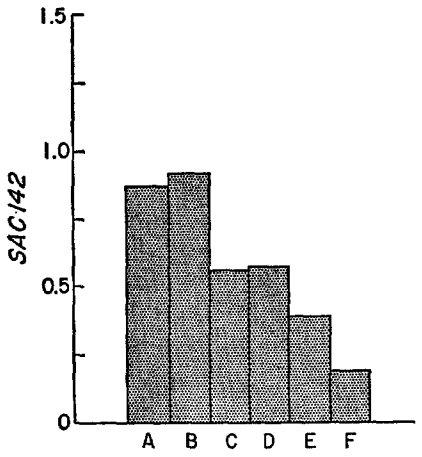

FIG. 10. The fluid phase destruction of $C2^{hu}$ by $C1^{hu}$ in the presence of homologous and heterologous C4. See text for content of reaction mixtures A through F.

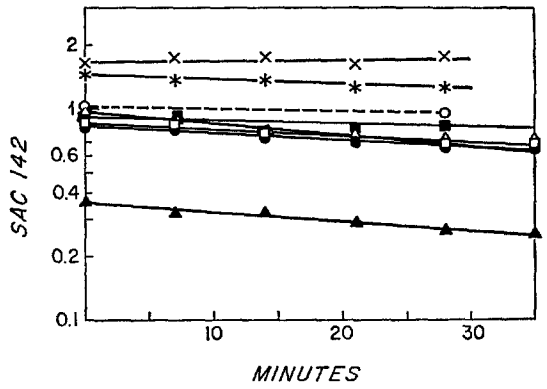

FIG. 11. The fluid phase destruction of $C2^{hu}$ by $C1^{hu}$ (●), and in the presence of homologous (▲), or heterologous C4 (✳), or equal mixtures of homologous and heterologous C4 (△, □). ○ refers to $C2^{hu}$ alone; × to $C2^{hu}$ incubated with $C4^{gp}$; and ■ to $C2^{hu}$ incubated with $C4^{hu}$.

sidual $C2^{hu}$ after incubation in buffer generated 0.96 SAC142; the presence of homologous or heterologous C4 with $C2^{hu}$ yielded the same or an increased number of SAC142. Exposure of the $C2^{hu}$ to $C1^{hu}$ alone reduced the sites produced to 0.65. $C1^{hu}$ in the presence of homologous C4 reduced the $C2^{hu}$ so that

only 0.26 SAC142 was generated, whereas the substitution of heterologous C4 protected C2 as indicated by the formation of 1.3 SAC142. The mixture of homologous and heterologous C4 afforded the same partial protection irrespective of the order of addition.

DISCUSSION

The present studies demonstrate that in the fluid phase $C4^{hu}$ is highly susceptible to inactivation by $C1^{hu}$, whereas $C2^{hu}$ is relatively resistant unless $C4^{hu}$ is also present in the mixture. These results extend the early observations of Lepow et al. (1) that the activation of C1 is associated with the capacity to inactivate C4 and to a lesser degree C2 in the pseudoglobulin fraction of human serum. In these early experiments no attempt was made to examine the action of C1 on C2 alone; thus the contribution of C4 has not been previously assessed in fluid phase mixtures.

Six units of $C1^{hu}$ were capable of inactivating 75% of 4 units of $C4^{hu}$ in 20 min (Fig. 1), whereas approximately 850 units of $C1^{hu}$ were required to produce a similar inactivation of 4 units of $C2^{hu}$ in the same time period (Fig. 3). Kinetic experiments again revealed the susceptibility of C4 to inactivation by small amounts of C1, 0.6–0.8 units (Fig. 2); whereas inactivation of 15 units of $C2^{hu}$ was negligible with 50 units of $C1^{hu}$ and required 500–5000 units for a striking effect (Fig. 4). Even at 5000 units, there was a lag phase to the action of $C1^{hu}$ on $C2^{hu}$ (Fig. 4) which was not apparent when inactivation of $C4^{hu}$ was examined (Fig. 2).

When 50 units of $C1^{hu}$ were interacted with $C2^{hu}$ in the presence of $C4^{hu}$, an increasing capacity to destroy C2 was demonstrable (Fig. 6). In the presence of $C4^{hu}$, the inactivation of $C2^{hu}$ by $C1^{hu}$ no longer exhibited a lag phase (Fig. 5). Instead there is an initial rapid disappearance of functional C2 in an interval of less than 5 min and comparable to that previously occupied by the lag phase (Figs. 4–6). After completion of the rapid phase of inactivation, $C2^{hu}$ inactivation proceeds at a rate comparable to its loss by the action of $C1^{hu}$ alone. The magnitude of the rapid phase inactivation of $C2^{hu}$ by $C1^{hu}$ in the presence of $C4^{hu}$ is related to the supply of $C4^{hu}$. As shown in Fig. 6, 4.6 units of $C4^{hu}$ permit approximately 30.5% inactivation; 13.2 units, 43% inactivation; 41.6 units, 60% inactivation; and 125 units, 84% inactivation of 1.5 units $C2^{hu}$ by 50 units of C1 within 5 min.

The finding that the enhanced inactivation of $C2^{hu}$ by $C1^{hu}$ in the presence of $C4^{hu}$ occurs within the first few minutes of interaction suggested a dependence upon the presence of hemolytically active $C4^{hu}$. Accordingly, an experiment was conducted in which $C1^{hu}$ and $C4^{hu}$ were interacted for varying time intervals prior to the addition of this mixture to hemolytically active $C2^{hu}$. The hemolytically active $C4^{hu}$ was inactivated (C4i) within 5 min of $C1^{hu}$ exposure, yet the reaction mixture $C1^{hu}$-$C4i^{hu}$ at that time and at various intervals thereafter was fully active in destroying C2 (Fig. 7). Indeed the $C1^{hu}$-$C4i^{hu}$

mixtures had slightly greater inactivating capacity than the initial $C1^{hu}$-$C4^{hu}$ mixture. Thus, the enhancement effect of $C4^{hu}$ on the action of $C1^{hu}$ on $C2^{hu}$ occurs during the period of $C4^{hu}$ inactivation and is manifested fully when all the native C4 has been converted to C4i.

The classical studies of Mayer and coworkers (6, 21) have clearly demonstrated that cell-bound first component, EAC1, does not uncover the binding site of C2 unless C4 is already fixed to the cell. Subsequently, Becker demonstrated (7) that the esteratic site of C1 must be intact for the EAC14 intermediate to interact with C2 to achieve the hemolytically active EAC142 state. Müller-Eberhard has presented evidence that the state of the fourth component in the hemolytically active intermediate is C4i (9, 10). Thus, it was important to demonstrate that the enhanced fluid phase inactivation of $C2^{hu}$ by $C1^{hu}$ in the presence of $C4i^{hu}$ occurred only when the esteratic site of $C1^{hu}$ was intact. $C1^{hu}$ inactivated by DFP had no effect on hemolytically active $C2^{hu}$ even in the presence of C4i (Fig. 8). When the C1 INH was employed to inhibit $C1^{hu}$, the $C4^{hu}$ in the reaction mixtures was inactivated, but there was no enhanced destruction of $C2^{hu}$ (Table I). These studies of DFP-inactivated or C1 INH–blocked first component reveal that the fluid phase inactivation of $C2^{hu}$ by $C1^{hu}$-C4i mixtures requires hemolytically active C1. These same experiments (Fig. 8 and Table I) demonstrate that the disappearance of hemolytically active $C2^{hu}$ from the reaction mixture on addition of $C4^{hu}$ or $C4i^{hu}$ is not explained by some direct effect of $C4^{hu}$ or $C4i^{hu}$ on $C2^{hu}$. The enhanced inactivation of $C2^{hu}$ by $C4^{hu}$ or $C4i^{hu}$ occurs only in the presence of enzymatically active $C1^{hu}$ and not when the esteratic site of $C1^{hu}$ is blocked by C1 INH or DFP. Further, in the experiment depicted in Fig. 6, which involved the addition of increasing amounts of $C4^{hu}$ to a limited amount of $C2^{hu}$, the loss of $C2^{hu}$ was related to the supply of $C4^{hu}$ only when C1 was present; there was no reduction in the hemolytic $C2^{hu}$ on addition of increasing amounts of $C4^{hu}$ alone.

It has been previously demonstrated by Nelson (22) and confirmed by Austen and Russell (23) that the cellular intermediate $EAC1^{gp}4^{gp}$ will not interact with $C2^{hu}$ so as to achieve a hemolytically active EAC142 intermediate. It was therefore of interest to examine the action of $C1^{gp}$ on functionally pure $C4^{hu}$ and $C2^{hu}$ and the effect of $C4^{gp}$ on the action of $C1^{hu}$ on $C2^{hu}$. At comparable hemolytic units, $C1^{gp}$ inactivates $C4^{hu}$ (Fig. 1) or $C2^{hu}$ (Fig. 3) to a much lesser extent than can be achieved with $C1^{hu}$. Not only did $C4^{gp}$ fail to enhance the action of $C1^{hu}$ on $C2^{hu}$ (Fig. 9), but it actually protects the $C2^{hu}$ from inactivation by $C1^{hu}$ (Figs. 10, 11). Although this protection could represent the failure of $C1^{hu}$ to utilize $C4^{gp}$, the finding that heterologous C4 interferes with the action of a $C1^{hu}$-$C4i^{hu}$ mixture on $C2^{hu}$ indicates interference at the latter step as well.

The enhanced disappearance of functionally active $C2^{hu}$ by the combined action of $C1^{hu}$ and $C4i^{hu}$ could involve either a change in the enzyme specificity or an alteration of substrate susceptibility. Mayer et al. (24) have demonstrated

the binding of C2 to cell-bound C4, presumably C4i; and Müller-Eberhard et al. (9) have demonstrated the formation of C4i-C2 complexes by physicochemical techniques. Thus it is possible that C4i alters C2 so as to present a more susceptible substrate to the C1 enzyme. Alternatively or in addition, $C4^{hu}$ may, through an allosteric action on $C1^{hu}$, uncover a specificity for native $C2^{hu}$. Irrespective of the mechanism, a role of $C4^{hu}$ is specifically to enhance the action of $C1^{hu}$ on $C2^{hu}$ in the fluid phase.

SUMMARY

The fluid phase inactivation of $C2^{hu}$ by $C1^{hu}$ is markedly enhanced by the presence of $C4^{hu}$. The enhancement is afforded by C1 inactivated $C4^{hu}$, namely $C4i^{hu}$, and requires the simultaneous presence of enzymatically active C1. Heterologous C4 of guinea pig origin protects $C2^{hu}$ from the inactivation by $C1^{hu}$. Thus, in both the fluid phase and on the cellular intermediate, $C4i^{hu}$ is essential to the specific action of $C1^{hu}$ on $C2^{hu}$. It is possible that C4i alters C2 so as to present a more suitable substrate to the C1 enzyme or that C4i acts on the C1 to uncover a specificity for native C2.

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