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

II. UNMASKING BY $C4i^{hu}$ OF $C\bar{1}^{hu}$ SPECIFICITY FOR $C2^{hu}$*

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It has been previously demonstrated (1) that the fluid phase inactivation of human second component of complement (C2) by activated human first component ($C\bar{1}$) is markedly enhanced in the presence of homologous fourth component (C4), whereas heterologous C4 is inhibitory[1]. This process was found to involve enzymatically active $C\bar{1}$ and the hemolytically inactive product of its action on C4, C4i. Since it is established that the $C\bar{1}s$ subunit of the $C\bar{1}$ macromolecule is directly capable of altering the electrophoretic mobility and hemolytic activity of C4 (2) and C2 (3, 4), the enzymatic site for C2 must be somewhat masked in the intact $C\bar{1}$ molecule. The present studies suggest C4i interacts directly with $C\bar{1}$ to uncover the active site for C2.

Materials and Methods

Sheep erythrocytes were processed as previously reported (5). Veronal-buffered saline, pH 7.5, 0.145 M, contaning 0.1% gelatin or 0.1% bovine serum albumin, 0.00015 M Ca^{++} and 0.0005 M Mg^{++} (GVB^{++} or $SAVB^{++}$)[2], dextrose-Veronal buffer ($DGVB^{++}$), and 0.01 M

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[1] The nomenclature used conforms to that agreed upon by the World Health Organization (1968, *Bull. World Health Organ.* **39**: 935). 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, 9. Fragments of individual components are subscripted with letters. The activated state of a component is signified by a bar above the component number. In addition to this convention the species of origin of a given component can be indicated by a superscript (hu, human; gp, guinea pig); in this manuscript components of human origin in the fluid phase are not designated, while the superscript is employed for components of other origins and for all cellular intermediates. 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.

[2] Other abbreviations used in this paper: $C\bar{1}INH$, human $C\bar{1}$ inhibitor; CM-cellulose, carboxymethyl-cellulose; DEAE-cellulose, diethylaminoethyl-cellulose; DFP, diisopropyl fluorophosphate; DGVB, dextrose-Veronal buffer; DSAVB, dextrose-bovine serum albumin-Veronal buffer; EDTA, disodium ethylenediaminetetraacetate; GPC-EDTA, guinea pig complement in 0.04 M EDTA; GVB, Veronal-buffered saline with 0.1% gelatin; SAVB, Veronal-buffered saline with 0.1% bovine serum albumin.

or 0.04 M disodium ethylenediaminetetraacetate (EDTA) were prepared in the usual fashion (6). The method of preparation of diethylaminoethyl (DEAE)- or carboxymethyl (CM)-cellulose and Sephadex G-200, and the buffers used in chromatography were previously described (6). Diisopropyl fluorophosphate (DFP), molecular weight 184.15, was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Sodium polyanetholsulfonate, Liquoid, lot No. 101028 from Hoffman LaRoche, Inc., Nutley, N.J. containing 10,000 units/g was diluted in saline and $SAVB^{++}$, instead of GVB^{++}, because a heavy precipitate formed when gelatin was present in the buffer.

Preparation of Human and Guinea Pig Components of Complement.—

Human first component and its subunits: $C\bar{1}$ was prepared by low ionic strength precipitation at pH 7.5 as described (6), and was further purified by Sephadex G-200 gel filtration (7). Unactivated C1, obtained either by low ionic strength precipitation at pH 5.5 as described (8) or from whole human serum, was purified by Sephadex G-200 gel filtration at high ionic strength, 0.9 M NaCl, in the presence of thrice crystallized soybean trypsin inhibitor, 100 mg/ml. The C1 activity was contained in the elution volume corresponding to the first protein peak.

The isolation (8) of the three subunits C1q, C1r, and C1s, was accomplished by chromatography on DEAE-cellulose of a euglobulin fraction of normal human serum obtained at pH 7.5 to yield $C\bar{1}$, or at pH 5.5 to maintain C1 in the unactivated state. The euglobulin precipitates were washed with cold 0.04 M NaCl in 0.005 M phosphate buffer, pH 7.5, or with 0.02 M acetate buffer, pH 5.5, resuspended in 0.3 M NaCl containing 0.001 M EDTA, and centrifuged. The supernatant fluid containing C1 or $C\bar{1}$ was dialyzed against three changes of 0.08 M NaCl in phosphate buffer, pH 7.5, with 0.001 M EDTA and centrifuged in the cold at 1935 *g*. 5 ml of supernatant were applied to a 28 × 2.5 cm DEAE-cellulose column equilibrated at the same pH and ionic strength. After a wash with three volumes of the starting buffer, a linear gradient of increasing salt concentrations from 0.08 M to 0.5 M NaCl was applied. C1q was present in the effluent immediately after the main protein peak; C1r eluted at 0.18 M and C1s at 0.35 M relative NaCl. Fractions containing C1q, C1r, and C1s were pooled, concentrated approximately 10 times in an Amicon apparatus membrane UM-10, and stored at −70°C with 0.001 M EDTA.

Human fourth component: C4, prepared according to Nelson et al. (6), was further purified by Pevikon block electrophoresis in barbital buffer at pH 8.6 (9). The block was run 24 hr in the cold at 400 volts and cut into twenty-one 1.2 cm sections, numbered from the origin. C4 was eluted in 0.15 M phosphate buffer, pH 7.4, from fractions 11–15. The eluates were pooled and concentrated in an Amicon apparatus with a membrane UM-10. The recovery represented 10% of the C4 activity in the starting whole human serum. This C4 preparation gave a single line with anti-human serum on analysis by the Ouchterlony technique, and two bands in acrylamide gel electrophoresis corresponding to C4 and C4i.

Human and guinea pig second component: C2 from both species was isolated by column chromatography on DEAE and CM-celluloses as described (1).

Human $C\bar{1}$ inhibitor ($C\bar{1}INH$): The $C\bar{1}INH$ was isolated from human serum according to procedures described (10).

Cellular Intermediates of the Hemolytic System and Titration of Components of Complement.—

Titrations of C1, C4, and C2 were performed with $EAC4^{hu}$ (11), $EAC1^{gp}$ (12), and $EAC1^{gp}4^{hu}$, (13, 14) respectively.

The C1 subunits, C1q, C1r, and C1s were examined for C1 hemolytic activity after recalcification of the individual column fractions with 0.005 M $CaCl_2$. A significant amount of hemolytic activity, presumably due to $C\bar{1}s$ (15), was present in the material eluted with 0.35 M NaCl when $C\bar{1}$ was used as starting material. When C1 was used, hemolytic activity

was obtained only upon recombination of the materials present in the eluate, the 0.18 M, and the 0.35 M fractions; the fractions were labeled C1q, C1r, and C1s, respectively, as described by Lepow et al. (8). C1q, C1r, and C1s were measured by serial dilutions of one of the subunits in the presence of excesses of the remaining two by the capacity to convert $EAC4^{hu}$ to $EAC1^{hu}4^{hu}$, as determined by lysis of this cellular intermediate when $C2^{gp}$ and guinea pig complement in EDTA were added sequentially.

RESULTS

Cellular and Fluid Phase Characteristics of the Unactivated First Component (C1).—

Three samples of C1 with approximately 25,000 units/ml were diluted 1:10,000, 1:20,000, and 1:40,000 in 20 ml $DGVB^{++}$ and prewarmed at 30°C. At zero time 20 ml of prewarmed

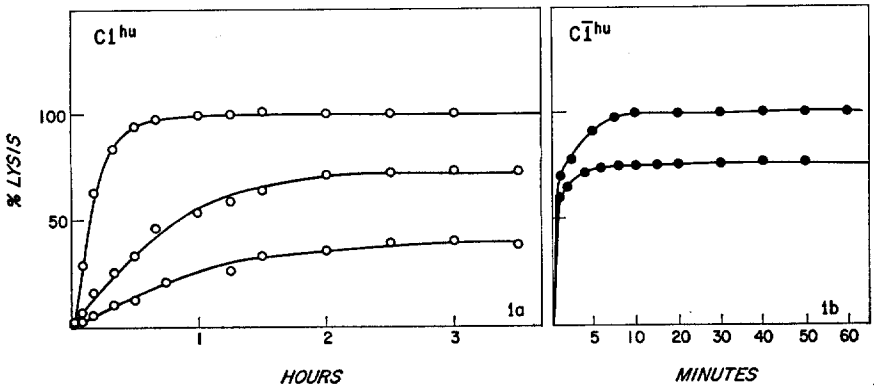

FIG. 1*a*. The kinetics of interaction of various dilutions of C1 with $EAC4^{hu}$.
FIG. 1*b*. The kinetics of interaction of various dilutions of $C\bar{1}$ with $EAC4^{hu}$.

$EAC4^{hu}$, 1×10^8 cells/ml, were added to each dilution and, at specified intervals thereafter, 1 ml samples were transferred to 10 ml of ice cold $DGVB^{++}$. The cells were immediately sedimented by centrifugation in the cold and resuspended in 1 ml $DGVB^{++}$; 0.5 ml of $C2^{gp}$ containing 100 effective molecules/cell was added and the cells incubated at 30°C for 10 min. 0.5 ml of 0.01 M EDTA and 1 ml of a 1:15 dilution of guinea pig complement in 0.04 M EDTA (GPC-EDTA) were then added and the cells incubated for 60 min at 37°C. The reaction volume was brought to 7.5 ml with ice cold saline and the per cent hemolysis determined (11).

Fig. 1a shows the generation of SAC14 by the three C1 dilutions. The highest C1 concentration permitted 100% lysis after 1 hr interaction with $EAC4^{hu}$, whereas the two limited dilutions of C1 required 2½ hr to approach plateaus of 37.5 and 72.5% hemolysis, respectively. In contrast, the same molecular concentrations of $C\bar{1}$ required only 7 min to facilitate maximal lysis (Fig. 1b).

The action of the C1 on C4 and C2 in the fluid phase was studied in the following experiment.

Three 2.5 ml samples of C2 containing 30 units/ml were mixed at 30°C with equal volumes of C4 containing 1000 units/ml. Immediately thereafter, one of the C2-C4 mixtures received 2.5 ml of C1 containing 200 units/ml, another received 2.5 ml of $C\bar{1}$ containing 200 units/ml, and the last received 2.5 ml $DGVB^{++}$. At zero time and at specified intervals thereafter, 0.5 ml samples were removed into 4.5 ml ice cold $DGVB^{++}$ for measurement of the residual hemolytically active C2. 0.5 ml of each diluted sample was added to 0.5 ml of $EAC1^{gp}4^{hu}$ cells, 1×10^8/ml. After incubation for 3 min at 30°C, 1.5 ml of a 1:22.5 dilution of GPC-EDTA were added, the mixtures incubated for 90 min at 37°C, and the per cent lysis determined (13).

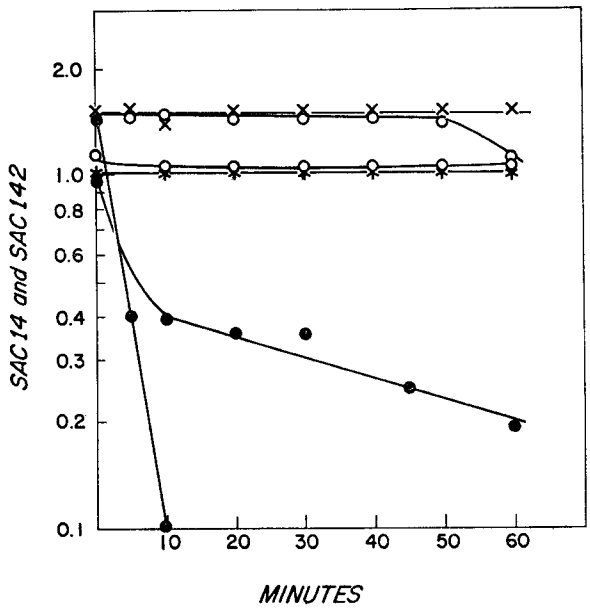

FIG. 2. Fluid phase inactivation of C4 and C2 by C1 (○), and $C\bar{1}$ (●). C4 (×) and C2 (*) incubated with buffer alone; see text for further details.

The original 1:10 dilution of each sample was further diluted 1:20 in $DGVB^{++}$ for a titration of the residual hemolytically active C4. To 0.5 ml of each diluted sample, 0.5 ml of $EAC1^{gp}$ cells, 1×10^8/ml, was added, and the reaction mixtures incubated for 15 min at 30°C. 0.5 ml $DGVB^{++}$ containing 100 $C2^{gp}$ effective molecules/cell was then introduced and the mixtures incubated for an additional 15 min at 30°C. After the addition of 0.5 ml of 0.01 M EDTA and 1.0 ml of 1:15 GPC-EDTA, the samples were incubated at 37°C for 60 min, and the per cent lysis determined (12).

Fig. 2 shows the residual C2 in each C2-C4 reaction mixture as measured by the formation of SAC142. The C2 in the C2-C4 mixture, incubated with buffer alone, formed 1.0 SAC142 and remained constant throughout the 60 min incubation. There was no decrease in C2 activity in the C2-C4 mixture incubated with C1; 1.0 SAC142 was formed even at the end of 60 min incubation.

In contrast when $C\bar{1}$ was present, there was a progressive loss of the capacity to form SAC142 such that only 0.2 SAC142 were produced by the 60 min sample. The residual C4 in the C2-C4 mixture incubated with $DGVB^{++}$ yielded 1.55 SAC14. In the presence of C1 only 20% of the C4 was inactivated in 60 min, whereas 98% was destroyed after 10 min of incubation with $C\bar{1}$.

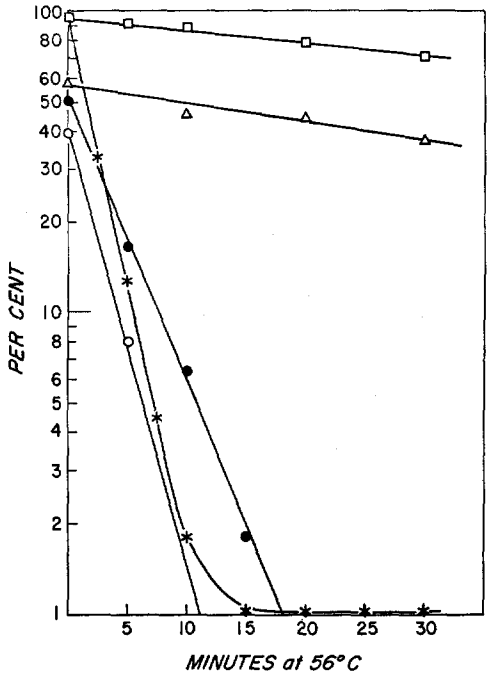

FIG. 3. The effect of heating $C\bar{1}$ at 56° for varying intervals on its hemolytic activity (*), interaction with $C\bar{1}INH$(△), and capacity to destroy C4 (□), C2 (○), and C2 in the presence of C4i (●).

The Activity of Heat Inactivated $C\bar{1}$.—

19 ml of $DGVB^{++}$ were equilibrated at 56°C in a water bath and 1 ml of $C\bar{1}$ containing 30,000 units/ml was added. Immediately after mixing and at specified intervals, 2 ml samples were removed and rapidly cooled to 0°C. A sample of $C\bar{1}$ diluted in $DGVB^{++}$ and kept at 0°C was included as an unheated control. Each of these heated $C\bar{1}$ samples was diluted serially in $DGVB^{++}$ and tested for hemolytically active $C\bar{1}$ (11).

As shown in Fig. 3, the $C\bar{1}$ hemolytic activity decreased rapidly upon incubation at 56°C. 69% of the hemolytic $C\bar{1}$ was destroyed during the first 2.5 min incubation, and in 10 min only 2% of the original $C\bar{1}$ was detectable. The 15, 20, and 30 min samples had $C\bar{1}$ values below 1% of those obtained in the unheated sample.

Fluid phase destruction of C4 by heated and unheated $C\bar{1}$:

$C\bar{1}$ heated at 56°C for 5, 10, 20, and 30 min and a sample of unheated $C\bar{1}$ were identically diluted in $DGVB^{++}$ to contain 100 $C\bar{1}$ units/ml in the unheated sample. 0.5 ml of each of these samples, or 0.5 ml $DGVB^{++}$ were mixed with 0.5 ml of C4 containing 2000 units/ml. After a 30 min incubation at 30°C the residual hemolytically active C4 was measured in serial dilutions of each reaction mixture.

$C\bar{1}$ heated at 56°C for 10 min inactivated 88% of the C4 present in the mixture (Fig. 3) even though heating had reduced the $C\bar{1}$ hemolytic activity by 98%; after 30 min of heating at 56°C the $C\bar{1}$ preparation was still capable of destroying 74% of the C4.

Fluid phase depletion of $C\bar{1}INH$ by heated and unheated $C\bar{1}$:

1 ml samples of unheated $C\bar{1}$ and $C\bar{1}$ heated at 56°C for 5, 10, 20, and 30 min, that contained 1000 units/ml before heating, were incubated with 1 ml of $C\bar{1}INH$ 1000 units/ml, in GVB^{++}. After 30 min incubation at 30°C all samples received 0.01 ml DFP. The mixtures were further incubated at 30°C for 10 min, followed by dialysis against three 1000 ml GVB^{++} changes. Control samples of $C\bar{1}$, treated with DFP and dialyzed prior to exposure to the $C\bar{1}INH$, and $C\bar{1}INH$ incubated with buffer alone, were included. The residual $C\bar{1}INH$ was measured in all the mixtures by the transfer technique (10, 16). 0.5 ml of $EAC1^{gp}$, 1.25×10^8 cells/ml, in GVB^{++} were mixed with 0.5 ml of serial dilutions of each of the $C\bar{1}$–$C\bar{1}INH$ DFP-treated mixtures and with the control samples. After incubation at 30°C for 30 min, 0.5 ml of $EAC4^{hu}$, 1×10^8 cells/ml, were added, and the cell mixtures incubated at 30°C for 15 min to allow the transfer of uninhibited $C\bar{1}^{gp}$. The $EAC1^{gp}4^{hu}$ formed were detected by adding $C2^{gp}$ and GPC–EDTA at identical concentrations and conditions as described above for the titration of $C\bar{1}$. $C\bar{1}INH$ incubated with buffer, or DFP-inactivated $C\bar{1}$ prior to incubation, gave identical results, and were used to determine how much inhibitor was depleted by $C\bar{1}$ or by $C\bar{1}$ heated.

As shown in Fig. 3, $C\bar{1}$ at time zero removed 58% of the $C\bar{1}INH$ added to the reaction mixture; after heating $C\bar{1}$ at 56°C for 10 min 47% of the $C\bar{1}INH$ was removed, even though the $C\bar{1}$ preparation was essentially devoid of hemolytic activity.

Fluid phase survival of C2 in the presence of heated $C\bar{1}$ and C4i:

Duplicate samples of 1 ml unheated and heated $C\bar{1}$, 1000 units/ml, were each mixed with 1 ml $DGVB^{++}$ in one set of tubes and 1 ml C4, containing 200 units/ml, in the other. All the samples received 1 ml of C2 containing 120 units/ml. Control mixtures of 1 ml C2 with either 2 ml of $DGVB^{++}$, or 1 ml $DGVB^{++}$ and 1 ml C4 were included. The mixtures were incubated for 30 min at 30°C and the residual hemolytically active C2 was titrated.

While the $C\bar{1}$ destroyed 50% of the C2 in the presence of C4i and 40% in its absence, $C\bar{1}$ heated at 56°C for only 10 min was unable to inactivate C2 alone or in the presence of C4i (Fig. 3).

The Effect of Liquoid on the Fluid Phase Destruction of C4 and C2 by $C\bar{1}$.—

Duplicate samples of $C\bar{1}$ containing 350 units/ml were incubated for 10 min at 30°C either with 0.2 ml Liquoid 0.006 mg/ml, or with 0.2 ml dextrose-bovine serum albumin-Veronal

buffer ($DSAVB^{++}$). Each set was divided into 1.1 ml samples which received either 1 ml C4 containing 200 units/ml or 1 ml $DSAVB^{++}$. All four reaction mixtures received 1 ml C2 containing 40 units/ml. Two control samples of 1 ml C4 and 1 ml C2 were incubated either with 1.1 ml $DSAVB^{++}$ or with 1 ml $DSAVB^{++}$ and 0.1 ml Liquoid. After 30 min at 30°C the residual hemolytically active $C\bar{1}$, C4, and C2 were measured in serial dilutions of each reaction mixture.

As shown in Fig. 4, $C\bar{1}$ alone destroyed 27% of the C2 units present in the mixtures (I), and this destruction increased to 73.5% in the presence of C4 (J). Incubation of $C\bar{1}$ with Liquoid produced a 90% inhibition of the hemolytic activity of $C\bar{1}$ (B), and totally protected C2 from fluid phase destruction by

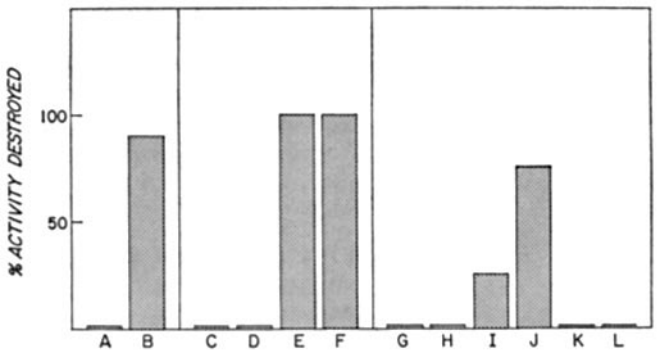

FIG. 4. The capacity of Liquoid to interfere with the hemolytic activity of $C\bar{1}$ and to protect C4, and C2 with and without C4 from fluid phase inactivation by $C\bar{1}$. The left hand panel depicts the effect of buffer (A) and Liquoid (B) on the $C\bar{1}$ hemolytic activity. The middle panel shows the loss of C4 activity after incubation with buffer (C), with buffer plus Liquoid (D), with $C\bar{1}$ (E), and with $C\bar{1}$ treated with Liquoid (F). The right hand panel indicates the C2 destroyed after incubation with buffer (G), with buffer and Liquoid (H), with $C\bar{1}$ (I), with $C\bar{1}$ in the presence of C4 (J), with $C\bar{1}$ pretreated with Liquoid (K), and with $C\bar{1}$ pretreated with Liquoid in the presence of C4 (L).

$C\bar{1}$ (K), even in the presence of C4i (L). In contrast, the presence of Liquoid did not protect C4; 100% of that present in the fluid phase was inactivated (F). C4 and C2 incubated with Liquoid (D, H) in the absence of $C\bar{1}$ yielded the same results as when these components were incubated with buffer (C, G). It was possible for Liquoid to protect C4 from fluid phase destruction by $C\bar{1}$ by reducing the ratio of $C\bar{1}$ to Liquoid. 95% of 215 C4 units were inactivated by 10 units of $C\bar{1}$, whereas in the presence of 0.01 mg Liquoid there was no C4 loss.

The Effect of C4 on the Behavior of $C\bar{1}$ Subunits.—

Fluid phase destruction of C4 by various subunit combinations:

0.5 ml of each of the C1 subunits in the combinations C1q, C1q–C1r, C1q–$C\bar{1}$s, C1q–C1r–$C\bar{1}$s, C1r, C1r–$C\bar{1}$s, and $C\bar{1}$s were brought to a total volume of 1.5 ml with $DGVB^{++}$ and

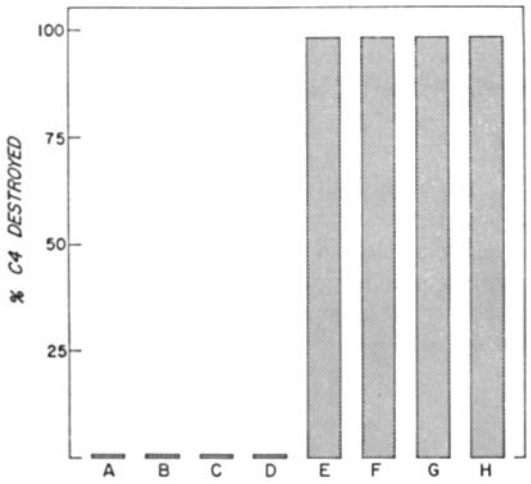

FIG. 5. The fluid phase destruction of C4 by various subunit combinations. C4 incubated with buffer alone (A), with C1q (B), C1r (C), C1q–C1r (D), $C\bar{1}s$ (E), $C\bar{1}s$–C1r (F), $C\bar{1}s$–C1q (G), and C1q–C1r–$C\bar{1}s$ (H).

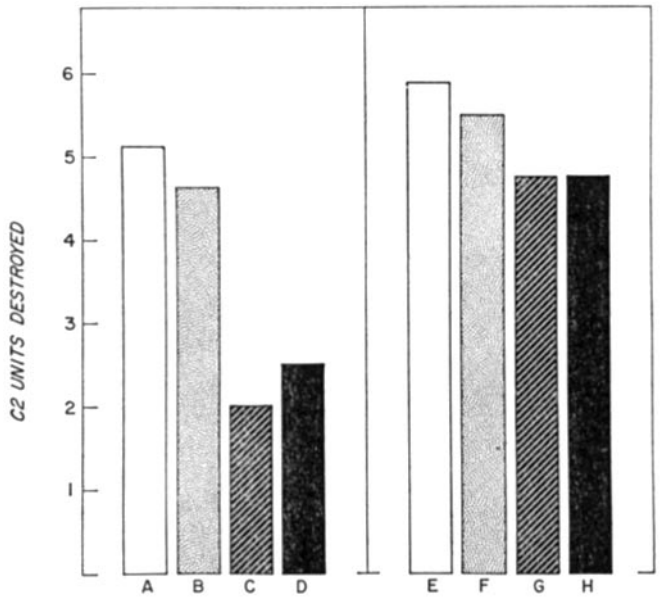

FIG. 6. Fluid phase destruction of C2 by various subunit combinations in the presence and absence of C4. C2 incubated with $C\bar{1}s$ (A), C1q–$C\bar{1}s$ (B), C1r–$C\bar{1}s$ (C), C1q–C1r–$C\bar{1}s$ (D), $C\bar{1}s$ in the presence of C4 (E), C1q–$C\bar{1}s$ in the presence of C4 (F), C1r–$C\bar{1}s$ in the presence of C4 (G), and C1q–C1r–$C\bar{1}s$ in the presence of C4 (H).

incubated at 30°C for 15 min. The dilution used for each subunit was equivalent to 50 $C\bar{1}$ units/ml when tested in the presence of an excess of the other two subunits. 0.5 ml of C4 containing 1600 units/ml was added to each of the 7 combinations and to 1.5 ml of $DGVB^{++}$. The samples were incubated for 30 min at 30°C and the residual hemolytically active C4 was measured in serial dilutions of each mixture.

The results depicted in Fig. 5 indicate that 98% of the C4 was destroyed in the fluid phase by all the samples in which $C\bar{1}s$ was present alone or in combination with C1q and C1r. C1q and C1r alone or mixed did not inactivate C4.

Fluid phase destruction of C2 by various $C\bar{1}$ subunit combinations in the presence and absence of C4:

Duplicate mixtures of 0.5 ml of C1 subunits in the combinations, C1q, C1q–C1r, C1q–$C\bar{1}s$, C1q–C1r–$C\bar{1}s$, C1r, C1r–$C\bar{1}s$, and $C\bar{1}s$, containing the equivalent of 100 $C\bar{1}$ units, were incubated at 30°C for 10 min in a total volume of 1.5 ml in $DGVB^{++}$. 0.5 ml of $DGVB^{++}$ was added to one set and 0.5 ml of C4 containing 200 units/ml to the other. After further incubation at 30°C for 10 min, 0.5 ml C2 at a final concentration of 10 units/ml was added. Two additional samples of C2 were incubated at the same concentration either with C4 and buffer or buffer alone. After 30 min at 30°C, the residual active C2 in each sample was determined.

The results (Fig. 6) indicate that $C\bar{1}s$ alone (A), or in combination with C1q (B), destroyed 5.2 and 4.7 C2 units, respectively; while the C1r-$C\bar{1}s$ (C) combination or a mixture of the 3 subunits (D) inactivated only 2.0 and 2.6 units, respectively. When C4 was added to the $C\bar{1}s$ (E) or C1q–$C\bar{1}s$ (F) combination there was a slight increase in C2 destruction to 5.8 and 5.5 units. In the samples with C1r–$C\bar{1}s$ (G) and C1q–C1r–$C\bar{1}s$ (H) the presence of C4 resulted in a two-fold increase in the capacity to destroy C2, 4.8 units being destroyed in both instances. C1q and C1r alone or combined did not influence C2 in the fluid phase.

The dose response of $C\bar{1}s$ inhibition by C1r and the reversal afforded by C4 were studied in the following experiments.

Five 0.1 ml samples of $C\bar{1}s$ containing 160 units of $C\bar{1}$ were incubated with 1 ml $DGVB^{++}$ or four 1 ml samples of C1r ranging in concentrations from 50 to 400 units/ml. The mixtures were incubated 15 min at 30°C and 1 ml $DGVB^{++}$ was added. 1 ml samples were removed from each reaction mixture and added to 0.5 ml C2 containing 10 units/ml. A control sample of C2 was incubated with buffer alone. After 30 min incubation at 30°C the residual active C2 was measured.

The results described in Fig. 7 show that increasing concentrations of C1r in the mixtures are associated with a decreasing destruction of C2 by $C\bar{1}s$. $C\bar{1}s$ alone destroyed 39% of the C2 in the mixtures, while in the presence of the highest concentration of C1r only 1.5% of the C2 was lost.

0.5 ml combinations of $C\bar{1}s$ and C1r containing 200 units of each were mixed with 0.5 ml $DGVB^{++}$ or 0.5 ml of different concentrations of C4, 400, 200, and 100 units/ml, respectively.

A sample of $\overline{C1}s$ at the same concentration was incubated with buffer alone. 0.5 ml of each mixture was removed and added to 0.5 ml C2 containing 20 units/ml. As controls 0.5 ml samples of C2 were incubated either with 0.5 ml C4 or with 0.5 ml $DGVB^{++}$.

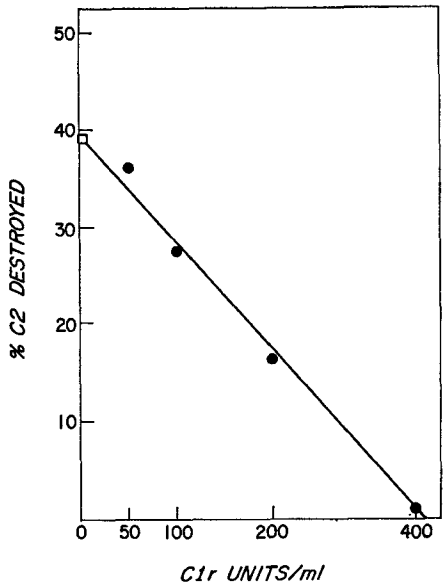

FIG. 7. Fluid phase destruction of C2 by $\overline{C1}s$ alone (□), and in the presence of increasing concentrations of C1r (●).

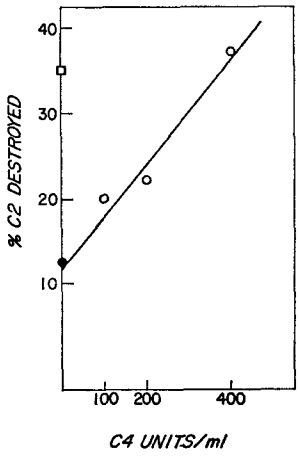

FIG. 8. Fluid phase destruction of C2 by $\overline{C1}s$–C1r alone (●), and in the presence of increasing concentrations of C4 (○); (□) indicates the action of $\overline{C1}s$ alone.

The results of this experiment are depicted in Fig. 8. $C\bar{1}s$ alone destroyed 34% of the C2 added, while the combination of $C\bar{1}s$ and C1r inactivated only 12.5%. In the presence of 100, 200, and 400 C4 units/ml, the C2 destruction increased to 20, 25, and 37%, respectively.

DISCUSSION

Although both activated (17) and unactivated (18) first component have been previously isolated, the comparative fluid phase activity of these preparations on their purified natural substrates C4 and C2 has not been reported. As shown in Fig. 2, the first component of complement isolated in its unactivated form, C1, did not inactivate C4 in the fluid phase during 60 min incubation, whereas material isolated in the activated form, $C\bar{1}$, destroyed all the C4 in the reaction mixture in the initial 10 min at 30°C. That C1 was indeed isolated in the unactivated state was documented by the appearance of hemolytic C1 activity after interaction with an EAC4 intermediate. 2½ hr were required to fully activate C1 as contrasted with immediate full hemolytic activity of the $C\bar{1}$ preparation (Fig. 1). As shown previously (1), fully activated $C\bar{1}$ converts C4 to C4i and concomitantly acquires the capacity to inactivate C2. Since it is known that the $C\bar{1}s$ subunit of the $C\bar{1}$ macromolecule is capable of altering the electrophoretic mobility and hemolytic activity of both C4 (2) and C2 (3, 4), the enzymatic site for C2 must be masked after activation of the intact C1 molecule has taken place.

The available evidence indicates that intact $C\bar{1}$ possesses a binding site (C1q) for immunoglobulin (19), an enzymatic site ($C\bar{1}s$) for C4 (2), and apparently a "receptor" through which C4i interacts to uncover a specificity for C2 (1). It was therefore of interest to determine which of these active sites was lost during heating $C\bar{1}$ at 56°C, so as to destroy hemolytic activity. It has been previously demonstrated that heat inactivated $C\bar{1}$ of the rabbit retains a binding site for EA as demonstrated by its capacity to protect EA and $EAC4^{gp}$ from lysis after the addition of native $C\bar{1}$ and the remaining components of the complement system (20). Similar experiments with heat inactivated human $C\bar{1}$ have also demonstrated a capacity to protect $EAC4^{hu}$ from lysis; in addition, EAC1 prepared with heat inactivated human $C\bar{1}$ removed C4 from the fluid phase without inactivating or fixing C2. Further documentation that the enzymatic site for C4 is intact in heat inactivated $C\bar{1}$ is presented in Fig. 3. Despite a 98% reduction in hemolytic activity, heat inactivated $C\bar{1}$ still destroyed C4 and interacted with $C\bar{1}$INH to remove the latter from the reaction mixture. On the other hand, the production and presence of C4i did not impart to heat inactivated $C\bar{1}$ the capacity to destroy C2. These findings are consistent with the view that the receptor for C4i in the $C\bar{1}$ molecule is heat labile, whereas the enzymatic site for C4 and the binding region for the immunoglobulin are relatively heat stable.

The highly cationic substance Liquoid was able to prevent the inactivation

of C2 by $C\bar{1}$ in the presence of C4i (Fig. 4). In addition, with appropriate ratios of Liquoid to $C\bar{1}$ it was also possible to inhibit the C4 destroying capacity of $C\bar{1}$. These data may reflect the fact that C4 is more susceptible than C2 as a substrate for $C\bar{1}$ and hence more difficult to protect from inactivation. The possibility also exists that Liquoid interferes with the capacity of C4i to unmask a C2 specificity by an effect other than inhibition of the active enzyme site. The studies of Naff and Ratnoff (21) indicated that Liquoid interfered with the esterolytic activity of the subunit C1r on *N*-acetyl-L-arginine methyl ester hydrochloride (AAME) but did not affect the activity of $C\bar{1}s$ against *N*-acetyl-L-tyrosine ethyl ester (ATEe). These findings are best explained by assuming that Liquoid inhibits the active enzymatic site by a steric effect, which is circumvented with a low molecular weight synthetic substrate but not with the natural substrates. Ratnoff and Naff further suggested that Liquoid inhibited the capacity of C1r to activate C1s to $C\bar{1}s$; however, the precise effect of Liquoid on activation remains to be examined, employing natural substrates, both in the fluid phase and on cellular intermediates.

The capacity (2, 3) of the subunit $C\bar{1}s$ to inactivate both C4 (Fig. 5) and C2 (Fig. 6) was confirmed. The introduction of other subunits into the reaction mixture had no effect on the inactivation of C4 by $C\bar{1}s$ (Fig. 5), while the presence of C1r with or without C1q significantly interfered with the capacity of $C\bar{1}s$ to inactivate C2. The presence of the C1r in a reaction mixture with $C\bar{1}s$ appears to mask the C2 specificity in a dose response fashion (Fig. 7), and thus creates a situation similar to that observed with $C\bar{1}$. As with the intact $C\bar{1}$ molecule (1), the presence of C4i uncovers the C2 specificity of a mixture of C1r and $C\bar{1}s$ in a dose response fashion (Fig. 8). These studies together with those of heat inactivation of $C\bar{1}$ suggest that the interaction of a heat labile receptor with C4i uncovers a C2 specificity masked by the presence of the C1r subunit in the $C\bar{1}$ macromolecule.

SUMMARY

It has been demonstrated that C1 isolated in the unactivated form fails to inactivate C4 or C2 in the fluid phase, while the activated molecule, $C\bar{1}$ rapidly converts C4 to hemolytically inactive C4i, but does not efficiently inactivate C2. The production and presence of C4i now confers on $C\bar{1}$ the ability to rapidly inactivate C2. After heating at 56°C, so as to destroy the hemolytic activity, heat inactivated $C\bar{1}$ is still capable of inactivating C4 but the presence of C4i no longer confers an ability to inactivate C2. Studies with the subunits of $C\bar{1}$–C1q, C1r, $C\bar{1}s$, indicate that the action of $C\bar{1}s$ on C2 can be inhibited by C1r and that this inhibition is reversed by the presence of homologous C4. These studies indicate that the interaction of C4i with a heat labile receptor conformation in $C\bar{1}$ uncovers a masked specificity for C2.

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