THE REACTION MECHANISM OF HUMAN C5 IN IMMUNE HEMOLYSIS*

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The fifth component of complement (C5) has been shown to play a key role in the biological actions of the complement system (1, 2). As yet unresolved, however, is the mode of action of C5 in immune cytolysis. Earlier studies in this laboratory led to the formulation of two alternative hypotheses for the mechanism of action of C5 (3). The "sequential action hypothesis" proposed that C5, C6, and C7 act in sequence with the formation of a cellular intermediate after each reaction step. The "functional unit hypothesis" proposed that C5, C6, and C7 act in concert as interdependent members of a single functional unit. It was not possible to choose between these hypotheses since experimental data in support of each was obtained.

This paper presents evidence which indicates that C5 may act through specific attachment to cells which have reacted with the first four complement components (EAC 1,4,2,3). It delineates the conditions of formation of a hemolytically active C5 intermediate complex, the physical fate of C5 in this reaction, and the occurrence of at least five different functional forms of the C5 molecule. Evidence will also be presented which shows that the cellular uptake of C5 and, hence, its hemolytic efficiency are greatly enhanced by C6 and C7. The simultaneous existence of two distinct pathways for C5 in immune hemolysis will be discussed.

Materials and Methods

Human Complement Components.—Highly purified C2 (4, 5), C3 (6), C4 (7), and C5 (6), and partially purified C1 (8), C6, and C7 (9) were isolated from human serum by published techniques. The activity of C2 was enhanced by oxidation with iodine as previously described (10). Preparations of C2, C3, C4, and C5 were trace-labeled with [125]iodine by the chloramine-T method (11) with full retention of hemolytic activity. The 12 preparations of ^{125}I-C5 used

in these studies had an average specific activity of 1.8×10^5 cpm/μg, a value which corresponds to 0.115 μCi/μg. Following Pevikon block electrophoresis, the labeled preparations showed an identical distribution of protein, radioactivity, and hemolytic activity. Additional tests performed to show that the labeled molecules were representative of the entire population of molecules in a given preparation included neutralization-competition tests and agglutination-inhibition tests. In the latter technique, C5 uptake by cells was estimated by a method independent of radioactivity measurement. EAC1,4,2,3,^{125}I-C5, bearing 1850 specifically bound C5 molecules per cell by radioactivity measurement, were added to dilutions of a monospecific antiserum to C5 in order to determine the agglutination end point. Next, an amount of purified, unlabeled C5 equal to the product of the number of radioactively labeled C5 molecules per cell times the number of cells, was added to dilutions of the antiserum which bracketed the previously determined end point. After a period of incubation, the EAC1,4,2,3,^{125}I-C5 were added. The agglutination titer was reduced by one twofold dilution. In simultaneous experiments incubation with twice the above amount of unlabeled C5 reduced the agglutination end point by two double dilutions. Since the amount of unlabeled C5 added was determined by nitrogen analysis, these studies indicated excellent agreement between the results obtained by radioactivity measurement and by immunochemical analysis.

Preparation of Serum Reagent Containing C6, C7, C8, and C9.—Guinea pig serum was incubated with an equal volume of cold 1 M KSCN for 16–18 hr at 4°C in order to inactivate C3, C4, and C5 in the serum (12). The mixture was then treated for 45 min at 37°C with a final concentration of 1.5×10^{-2} M hydrazine hydrate, a reagent which also inactivates C3, C4, and C5. After extensive dialysis at 4°C against veronal-buffered NaCl, pH 7.4, the treated serum was frozen. For use in the effective molecule titration of C5, the serum was thawed and diluted 1:100 in 0.04 M EDTA in veronal-buffered NaCl. This reagent produced a constant level of hemolysis of EAC1,4,2,3,5, bearing a limited number of C5 sites to a dilution of 1:200.

Labeling of Erythrocytes with Radioactive Chromium.—15 ml of sheep erythrocytes (E),[1] 5×10^8/ml in veronal-buffered NaCl, were incubated with 500 μCi of ^{51}Cr-sodium chromate (13) for 45 min at 37°C. The cells were then washed five times in veronal-buffered NaCl, pH 7.4, transferred to a clean tube, and washed again prior to use. The erythrocytes were indistinguishable from unlabeled E with respect to reactivity with antibody and C1, C2, C3, C4, and C5. Determinations of free hemoglobin by spectrophotometer and by ^{51}Cr-measurement gave identical values for percentage of lysis of EAC1,4,2, and EAC1,4,2,3 in the presence of the remaining components.

Preparation of Sheep Erythrocyte-Antibody-Complement Complexes.—The preparation of EAC1 (8), EAC1,4 (5), EAC1,4,2 (5), and EAC1,4,2,3 (14) has been described. EAC1,4,2,3 for C5 titration and uptake studies were prepared as follows. EAC1,4, bearing approximately 3000 specifically bound C4 molecules per cell, were formed by the addition to EAC1 of 200 effective molecules of C4 per cell (100 μg/5 $\times$ 10^9 EAC1). Following restoration of C1 (5), the EAC1,4 were reacted with 50 effective molecules of oxidized C2 per cell, a dose which is sufficient to saturate the bound C4 molecules (5). After incubation for Tmax time and washing, 500 μg of C3 per 5×10^9 cells were added. This amount of C3 results in the specific binding of approximately 25,000 C3 molecules per cell. EAC1,4^{oxy}2,3, bearing known numbers of C4,2 sites per cell, were prepared from EAC1,^{125}I-C4. Each lot of EAC1,^{125}I-C4, bearing a known number of radiolabeled C4 molecules per cell, was reacted sequentially with sufficient ^{oxy}C2 to saturate the C4 sites (5) and a constant excess of C3.

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[1] *Abbreviations used in this paper*: E, sheep erythrocytes; Tmax, time at which there is maximum formation of an intermediate cell-antibody–complement complex.

Effective Molecule Titration of C5.—EAC1,4,2,3 in EDTA-GVB (veronal-buffered NaCl, pH 7.4, containing 0.1% gelatin and 0.01 M EDTA [14]) at a concentration of 1.5×10^8/ml were dispensed in 0.2 ml portions in a series of tubes. Then, 0.2 ml of EDTA-GVB, followed by 0.2 ml of dilutions of C5, were added and the tubes incubated with shaking at 30°C. After a predetermined optimal period of reaction (Tmax), which was usually about 10 min, 2.4 ml of a 1:100 dilution of KSCN hydrazine-treated guinea pig serum in 0.04 M EDTA-GVB were added. After 1 hr of incubation at 37°C, the tubes were centrifuged and the optical density of the supernatant solutions was determined at 412 mμ on a Beckman DU spectro-photometer (Beckman Instrument Co., Fullerton, Calif.). Controls included, in duplicate, EAC1,4,2,3 plus KSCN-hydrazine guinea pig serum (control 1), a KCNS-hydrazine serum color blank (control 2) and a water lysate of the cells. The optical density of control 1 was subtracted from that of the experimental tubes, while the difference between the optical densities of controls 1 and 2 was subtracted from the 100% lysis value. The corrected reading for each experimental tube was then divided by the corrected 100% lysis value to obtain the proportion of cells lysed (y). The degree of lysis, when plotted against the dilution of C5, describes a curve which is concave toward the dose scale. This relationship may be converted to one of direct proportionality when the reciprocal of the negative natural logarithm of the unlysed cells, i.e. $1/-\ln(1-y)$ (16), is graphed arithmetically against the reciprocal of the dilu-tion of C5. As the $-\ln(1-y)$ value expresses the average number of effective C5 sites per cell (17), a $-\ln(1-y)$ value of unity indicates one C5 site or one effective molecule of C5 per cell. Multiplication of the reciprocal of the dilution producing a $-\ln(1-y)$ value of unity by the number of cells gives the number of effective molecules present in the sample containing C5.

Quantitation of Uptake of Radioactively Labeled C5.—Prior to use, crystalline human serum albumin (Behringwerke A. G., Marburg-Lahn, Germany) was added to the ^{125}I-C5 preparation to a final concentration of 1 mg/ml in order to prevent aggregation of the labeled preparation which occurs on pipetting. The preparations were then centrifuged for 20 min at 2000 g to re-move any preformed aggregates of C5. Uptake studies were performed under conditions identi-cal to those employed for the effective molecule titration of C5, except that the volume of all reagents was increased 2.5-fold. After completion of the lytic period, the tubes were centrifuged and the supernatant fluids were analyzed for released hemoglobin. The cells and stromata of each of the reaction mixtures were washed four times at 4°C, transferred to clean tubes, and analyzed for radioactivity in a well-type scintillation counter. In some experiments C5 uptake was terminated at Tmax by centrifugation and washing of the cells. The samples were then di-vided into two portions, one of which was added to the C6-9 reagent in order to determine the hemolytic activity of bound C5; the other was analyzed for radioactivity. Nonspecific uptake of C5 was determined in all studies from reaction mixtures identical to those employed for the titration of C5, except that E, EA, EAC1,4 or EAC1,4,2 were substituted for EAC1,4,2,3. Specific uptake was obtained by subtracting the values for nonspecific uptake from the total bound radioactivity. Nonspecific binding of C5 averaged 20% of the total radioactivity and thus was considerably higher than has been observed in studies with any of the other complement proteins. Although nonspecifically adsorbed C5 was firmly bound, cells bearing only nonspecifically bound C5 were not agglutinated by antisera to C5. A mol wt of 2×10^5 was used to calculate the number of bound C5 molecules. The percentage of uptake of C5 was calculated from the linear portion of a graph of [125]C5 input against specific ^{125}I-C5 uptake.

Determination of the Hemolytic Efficiency of Cell-Bound C5.—The supernatant fluids of the reaction mixtures used to determine the uptake of ^{125}I-C5 were saved and analyzed for released hemoglobin. The hemolytic values, expressed as $1/-\ln(1-y)$, obtained for the cells prepared from each dilution of ^{125}I-C5, were plotted arithmetically against the reciprocal of the number of specifically bound ^{125}I-C5 molecules per cell, as determined from the radio-active uptake values. The number of cell-bound C5 molecules necessary to produce one hemolytically effective molecule was obtained from the graph at a $-\ln(1-y)$ value of unity.

C5 Transfer Studies.—Varying numbers of washed EAC1,4,2,3,5, termed donor cells, were added to 0.2 ml of 1.5 × 10⁸/ml EAC1,4,2,3, termed recipient cells. In order to permit differentiation of the lysis produced by the two cell types, one of the cell types, generally the recipient cells, was labeled with ⁵¹Cr. The cell mixtures were centrifuged and resuspended in 50 μl of EDTA-GVB and, after 10 min of shaking at 30°C, 0.55 ml of EDTA-GVB and 2.4 ml of 1:100 C6-9 reagent were added. After 60 min at 37°C the reaction mixtures were centrifuged and the optical density and radioactivity of the supernatant fluids were determined. The percentage of lysis of the recipient cells was obtained by quantitating the release of radioactivity. From the spectrophotometrically and radioactively determined hemoglobin release, the percentage of lysis of the donor cells was calculated.

Neutralization-Competition Tests.—This technique was introduced by Stroud et al. (18) as an analytical method for detecting and studying hemolytically inactive products of C2. In the present study this test was used to detect hemolytically inactive C5 eluted from cells.
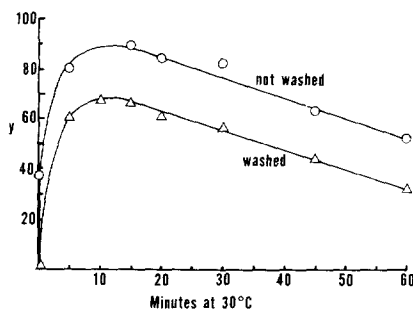

Fig. 1. Tmax of C5. Kinetics of formation of a C5 intermediate complex. Equal volumes of C5 (0.01 μg/ml) and EAC1,4,ᵒˣʸ2,3 (1.5 × 10⁸/ml) were incubated at 30°C. Duplicate samples were taken at intervals, one of which was added directly to the C6-C9 reagent (not washed), while the cells in the other sample were washed prior to addition to the lytic reagent (washed).

A preparation of C5 having an identical hemolytic activity to protein ratio in labeled and unlabeled forms was used to prepare EAC1,4,ᵒˣʸ2,3,5. The same dose, 1.4 × 10⁵ molecules per cell, of both forms of C5 was also added to EAC1,4 as a control for nonspecifically bound C5. These cell preparations were centrifuged after 45 min at 30°C, and the supernatant fluids were analyzed for eluted, hemolytically inactive C5. Varying amounts of the supernatant fluids were added to 2.32 effective molecules of C5. Next, a dilution of antibody to C5 capable of neutralizing the hemolytic activity of 1.05 effective molecules of C5 was added. After 60 min at 37°C, the mixtures were titrated for hemolytically active C5. An increase in the amount of active C5 indicated the presence of inactive C5 in the reaction mixture. The number of molecules of inactive C5 necessary to produce a given increase in free active C5 was determined by calculation, assuming that active and inactive C5 reacted equally well with anti-C5.

RESULTS

Formation of a C5 Intermediate Complex and the Fate of Fluid-Phase C5.— Since initial attempts to isolate a cellular intermediate bearing C5 activity led

to variable results, various parameters of the reaction of C5 with EAC1,4^{oxy}2,3 were analyzed. In order to characterize the kinetics of the reaction, a mixture of C5 and EAC1,4,^{oxy}2,3 was incubated at 30°C. Periodically, duplicate samples were removed, one of which was added directly to the C6-9 reagent, while the cells in the other sample were washed twice at 0°C prior to their addition to the lytic reagent. Fig. 1 shows the kinetics of formation and decay of a C5 intermediate complex.
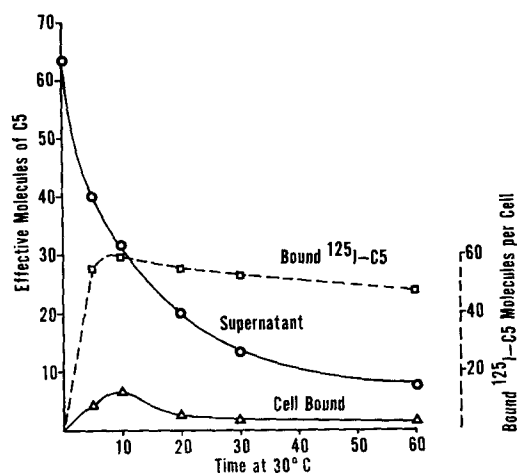

FIG. 2. Fate of C5 upon reaction with EAC1,4,2,3. Association of C5 binding with the formation of a hemolytically active C5 intermediate complex and inactivation of C5 in the fluid phase. Equal volumes of ^{125}I-C5 (0.46 μg/ml) and EAC1,4,^{oxy}2,3 (1.5 × 10^8/ml) were incubated at 30°C. Residual C5 activity was measured in the supernatant fluid of samples taken at intervals (supernatant), while the cells in each sample were washed and analyzed for cell-bound C5 radioactivity (bound ^{125}I-C5) and hemolytic activity (cell bound). Cell-bound hemolytic activity was measured indirectly: The washed cells from each sample were placed at 30°C and the loss of C5 activity as a function to time was determined. By extrapolation of the decay curve, the number of effective molecules of C5 per cell at the times of the original samples was obtained.

In order to determine whether the acquisition of C5 activity was accompanied by physical attachment of C5 to the cells, a similar experiment was performed with radiolabeled C5. Samples were taken at intervals and the cells in each of the samples, after four washes at 4°C, were examined for both cell-bound C5 radioactivity and hemolytic activity. The supernatant fluid of each sample was saved and subsequently titrated for residual C5 hemolytic activity. Examination of the results of this experiment (Fig. 2) shows that the acquisition of C5 activity by the cells was correlated with the uptake of ^{125}I-C5. In addition, maximal formation of EAC1,4,2,3,5 and maximal uptake of radioactively

labeled C5 occurred at the same time (Tmax). During the process of formation of EAC1,4,2,3,5, the hemolytic activity of unbound C5 in the fluid phase was rapidly lost. Since less than 10% of the C5 activity which disappeared from the fluid phase phase could be accounted for by cellular uptake, the loss must have been due to inactivation without binding.

The Role of $C\overline{4,2}$ and C3 in the Binding and Activation of C5.—In order to investigate the influence of the number of $C\overline{4,2}$ molecules per cell on C5 uptake, EAC1,^{125}I-C4,^{oxy}2,3, bearing known numbers of $C\overline{4,2}$ molecules per cell, were mixed with a single ^{125}I-C5 preparation. A linear relationship was found to prevail between the number of bound $C\overline{4,2}$ molecules per cell and the uptake of C5,
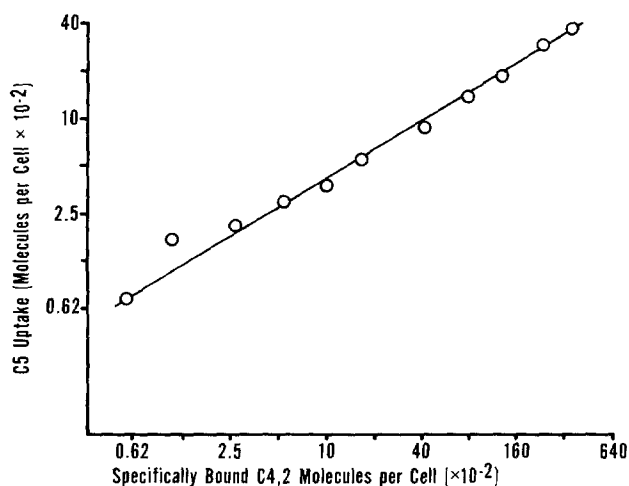

Fig. 3. Dependence of C5 uptake on the number of $C\overline{4,2}$ molecules per cell. The uptake of ^{125}I-C5 by EAC1,^{125}I-C4,^{oxy}2,3 was determined with cells which varied 1000-fold in the number of ^{125}I-$C\overline{4,2}$ molecules per cell.

when these results were expressed logarithmically (Fig. 3). Each 10-fold increment in the number of $C\overline{4,2}$ molecules per cell, over a 1000-fold range, produced a 4-fold increase in the uptake of C5. A similar linear relationship prevailed when the effective molecule titer of a single C5 preparation was determined with several batches of EAC1,^{125}I-C4,^{oxy}2,3, bearing known numbers of $C\overline{4,2}$ molecules per cell. In this instance, however, the resulting linear relationship showed that each 10-fold increase in the number of $C\overline{4,2}$ molecules per cell was reflected in a 25-fold increase in the effective molecule titer of the C5 preparation.

Essentially identical studies were performed with EAC1,4,^{oxy}2,^{125}I-C3, bearing constant numbers of $C\overline{4,2}$ molecules per cell (approximately 3000) and in-

creasing numbers of ^{125}I-C3 molecules per cell. A linear relationship existed between the number of C3 molecules per cell and the uptake and effective molecule titer of the C5 preparation. Each 10-fold increment in the number of C3 molecules per cell produced a 3-fold increase in the uptake of C5 and a 7-fold increase in its hemolytic activity.

It is apparent that a constant titer or uptake of C5 is not achieved even with very large numbers of $C\overline{4,2}$ and C3 molecules per assay cell. Therefore, in subsequent studies EAC1,4,2,3, bearing approximately 3000 $C\overline{4,2}$ molecules and 25,000 C3 molecules per cell, have generally been used.
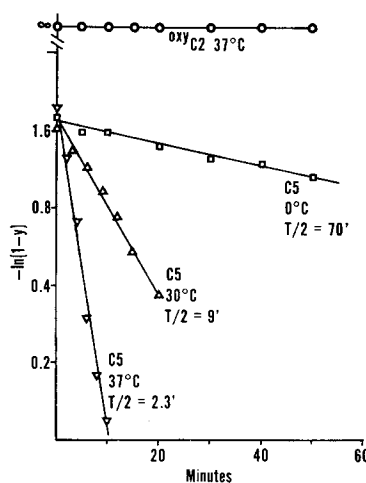

FIG. 4. Lability of the hemolytic activity of bound C5. EAC1,4,[oxy]2,3,5 were incubated at 0°C, 30°C, or 37°C and sampled at intervals for residual cell-bound C5 activity. Complete lysis was observed on testing the cells for [oxy]C2 activity throughout the period of study.

Lability of the Hemolytic Activity of Bound C5.—Fig. 4 shows the rate of loss of bound C5 activity on EAC1,4,[oxy]2,3,5 as a function of time and temperature. The half-life of bound C5 at 30°C in 20 determinations was 8–10 min, and at 37°C it was 2.3 min. In many experiments a second linear component of the decay curve was observed after 20–30 min of incubation at 30°C. This second phase, of unknown significance, had a half-life of 16–20 min at 30°C.

Is Decay of Cell-Bound C5 Activity Due to Dissociation of C5 Protein from EAC1,4,2,3,5?—Dissociation of C5 protein from EAC1,4,2,3,5 as the possible cause of the lability of bound C5 activity was explored using radiochemical techniques and the neutralization-competition method. The intermediate complexes EAC1,4,2,3,5, EAC1,[oxy]2,3,5 and EAC1,4,[oxy]2,3,5,6,7 were prepared with radioactively labeled C5; ^{125}I-C5 was also incubated with EAC1,4 as

a control for nonspecifically adsorbed C5. At Tmax the cells were centrifuged, washed, and placed at 30°C. The cells in samples taken at intervals were washed and the cell-bound radioactivity determined and corrected for nonspecifically bound C5. Very little loss of bound ^{125}I-C5 occurred over a 2 hr period (Fig. 5), a conclusion further substantiated by the finding of less than 10% of the total radioactivity in the supernatant fluid of the 2 hr sample. It should be noted that the functional status of C2 and the presence of C6 and C7 had no influence on the very slow elution of bound C5. C5 protein readily binds nonspecifically to
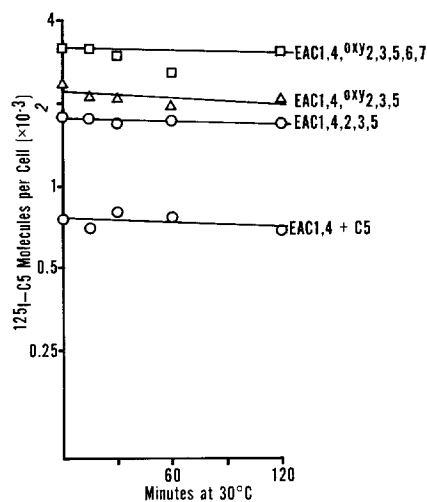

Fig. 5. Release of C5 from various intermediate complexes. Intermediate complexes bearing ^{125}I-C5 were incubated at 30°C; cells bearing nonspecifically bound C5 (EAC1,4 + C5) were also included. Samples were taken at intervals and the cells, after washing, were analyzed for bound radioactivity. Dissociation of either specifically or nonspecifically bound C5 was minimal.

cells, as shown by the high levels of uptake by EAC1,4. This form of C5 also remained firmly bound.

The neutralization-competition technique was employed in order to rule out a difference in dissociation behavior of labeled and unlabeled C5. In these experiments C5 cells were prepared with radioactive and nonradioactive C5. The two intermediates were comparable with respect to bound C5, as they gave identical end points with dilutions of antibody to C5. Measurements of cell-bound radioactivity showed 1930 C5 molecules per cell at the onset of the experiment and 1780 molecules per cell after 45 min of incubation, indicating a release of 8% of the bound ^{125}I-C5. Analyses of the supernatant fluids indicated that EAC1,4,^{oxy}2,3,[125]I-C5 released sufficient hemolytically inactive C5 in 45 min

to produce an increase of 0.29 effective molecules of active C5 in the neutralization-competition test, while the supernatant of cells prepared with unlabeled C5 produced an increase of 0.16 effective molecules of active C5. No release of nonspecifically bound C5 was detected. If it is assumed that native and cell-dissociated C5 react equally well with antibody to C5, the above values correspond respectively to the release of 490 and 240 molecules of labeled and unlabeled C5 per cell in 45 min at 30°C. Thus, the neutralization-competition test detected
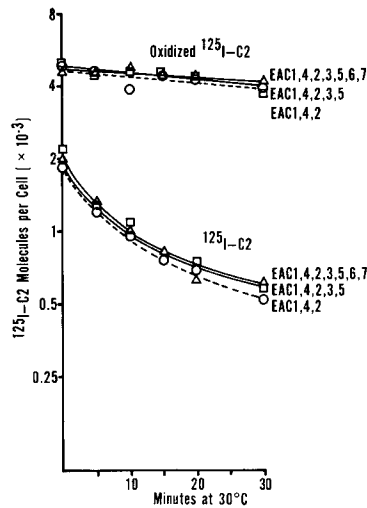

FIG. 6. Release of C2 from various intermediate complexes. Intermediate complexes bearing either native or oxidized ^{125}I-C2 were incubated at 30°C and periodically sampled for residual cell-bound C2 radioactivity. Oxidized C2 was released at a low rate from the intermediate complexes tested. Native C2 dissociated from each of the three complexes with an initial half-time of 10 min.

release of 25% of the total of bound ^{125}I-C5 in 45 min incubation at 30°C and showed that there was little difference between labeled and unlabeled C5 in this regard. These studies show that dissociation of C5 from C5 cells does occur, but at a rate which is not sufficiently rapid to explain the rapid loss of C5 hemolytic activity from EAC1,4,2,3,5.

Is Decay of Cell-Bound C5 Activity Due to Dissociation of C2 from EAC1,4,2, 3,5?—The complexes EAC1,4,2, EAC1,4,2,3, EAC1,4,2,3,5, and EAC1,4, 2,3,5,6,7 were prepared separately with ^{125}I-C2 in both untreated and oxidized forms. The cell preparations were washed, placed at 30°C, and periodically sampled. Fig. 6 reveals that oxidized ^{125}I-C2 was firmly bound to all cell types examined. In the same experiment unoxidized C2 was found to elute with an

initial half time of 10 min at 30°C which coincides with its hemolytic half time at this temperature. Thus, the lability of bound C5 activity could not be explained by dissociation of C2. Fig. 7 reveals that this lability was also not due to alteration of the functional status of C2.

Molecular Size of Cell-Bound C5.—To determine the approximate size of cell-bound C5, cells bearing ^{125}I-C5 were incubated in a concentrated suspension for 3 hr at 37°C and the supernatant fluid, containing about 30% of the original cell-bound C5, was analyzed by ultracentrifugation in a sucrose gradient. As shown in Fig. 8, native and cell-dissociated C5 exhibited a comparable sedimentation behavior.
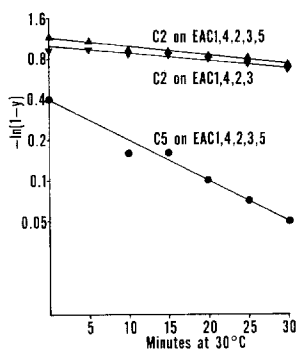

FIG. 7. Effect of C5 on cell-bound ^{oxy}C2. EAC1,4,^{oxy}2 were prepared with limited ^{oxy}C2 (1.1 effective molecules/cell) and the cells were subsequently exposed to an excess of C3 (2 × 10^5 molecules/cell) and C5 (5.6 × 10^4 molecules/cell). The cells were then washed, placed at 30°C, and periodically sampled for residual C2 activity. The slow rate of loss of bound ^{oxy}C2 activity was independent of the presence or absence of C5 on the cells.

Hemolytic Efficiency of C5.—In order to analyze the binding efficiency of C5, effective molecule titrations were carried out with ^{125}I-C5 and following completion of the lytic period the cells and stromata were washed and analyzed for ^{125}I-C5 uptake. Fig. 9 shows the effective molecule titration of a C5 preparation obtained with EAC1,4,^{oxy}2,3 bearing approximately 3000 $C\overline{4,2}$ sites per cell. A -ln(1 − y) value of unity occurred at a 1:24,000 dilution; however, when the cells were washed at Tmax prior to the addition of C6-9, ten times more C5 was required to produce the same degree of lysis. Fig. 10 shows the relationship between ^{125}I-C5 input and uptake obtained in this experiment. The specific uptake of C5 obtained from the linear portion of the curve was 0.88% of input. When binding of C5 was determined in the absence of the C6-9 reagent, the uptake was only 0.18%. The results of six uptake experiments are shown in Table I. Even with very large numbers of $C\overline{4,2}$ and C3 molecules per cell, uptake was maximally 4% of input.

Fig. 11 shows the effective molecule titration as a function of the number of bound C5 molecules per cell. A -ln(1 — y) value of unity occurred with five specifically bound molecules of C5 per cell. When C5 was bound in the absence of C6-9, -ln(1 — y) = 1 occurred with 12 molecules per cell. Table I summarizes
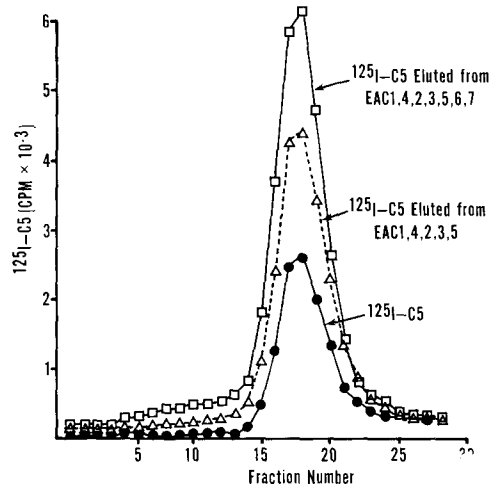

Fig. 8. Sucrose density gradient ultracentrifugation of C5 dissociated from C5 cells. EAC1,4,^{oxy}2,3,5 and EAC1,4,^{oxy}2,3,5,6,7, prepared with ^{125}I-C5, were incubated in concentrated suspensions for 3 hr at 37°C. After centrifugation, the supernatant fluids were analyzed by ultracentrifugation. Fraction 1 represents the bottom of the centrifuge tube.
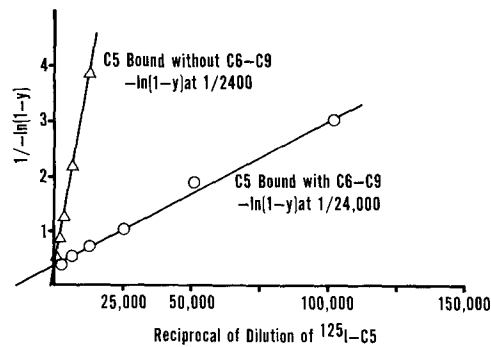

Fig. 9. Effective molecule titration of a preparation of ^{125}I-C5 (No. 5). The protein concentration of this preparation was 0.720 mg/ml. The titer was reduced 10-fold when the titration was interrupted by a series of washes after the C5 reaction step (bound without C6-C9).

the results of six studies of the hemolytic efficiency of bound C5. An average of seven bound C5 molecules were required to cause lysis of a cell, regardless of whether C5 was bound in the presence or absence of C6-9. It is apparent, therefore, that the effect of the C6-9 reagent is confined to increasing the uptake of C5, not to the hemolytic efficiency of bound C5.
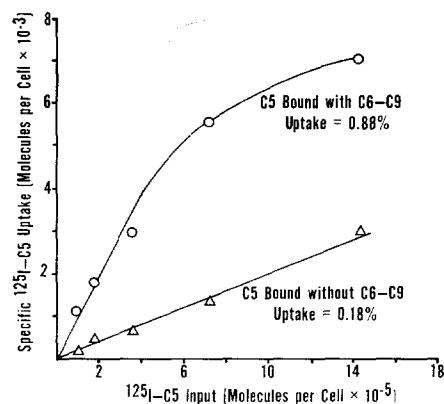

Fig. 10. Relationship between input of ^{125}I-C5 (No. 5) and its specific cellular uptake by EAC1,4,^{oxy}2,3. The C5 preparation and cells are the same as those shown in Figs. 9 and 11. Uptake of C5 was enhanced approximately 5-fold by the presence of C6-C9.

TABLE I

Efficiency of Binding and Hemolytic Efficiency of Bound ^{125}I-C5 EAC1,4,^{oxy}2,3 + ^{125}I-C5

Experiment No.	C6-9 added later			C6-9 present		
	A % uptake of ^{125}I-C5	B Bound Mole-cule/Effective Molecule	C Hemolytic efficiency 100/A × B	A % uptake of ^{125}I-C5	B Bound mole-cule/effective molecule	C Hemolytic efficiency 100/A × B
1	0.02	9	45000	0.6	12	2000
2	0.2	12	6000	0.9	5	560
3	0.8	4	500	3.0	5	166
4	1.1	8	730	3.5	5	143
5	0.5	1	200	3.3	3	90
6	0.5	1	200	3.9	3	77

Effect of C6 and C7 on the Binding of C5.—As indicated above (Fig. 10, Table I), the uptake and, hence, the hemolytic activity of C5 were markedly reduced when the reaction of C5 with EAC1,4,2,3 was carried out in the absence of C6-C9. Because of the known physicochemical interaction between C5, C6, and C7 in free solution (2),[2] the effect of C6 and C7 on the uptake of ^{125}I-C5 was

[2] Götze, O., and H. J. Müller-Eberhard. Lysis of erythrocytes by complement in absence of antibody. *J. Exp. Med.* In press.

explored. As shown in Fig. 12, the presence of C6 and C7 increased the uptake of ^{125}I-C5 in direct proportion to the amount of these components offered. In this experiment the maximal increase of C5 uptake produced by C6 and C7 was approximately fourfold.
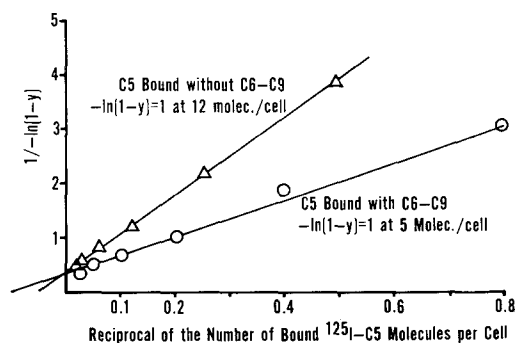

Fig. 11. Hemolytic efficiency of cell-bound ^{125}I-C5 (No. 5). Relationship between effective molecules of ^{125}I-C5 and the number of specifically bound ^{125}I-C5 molecules per cell.
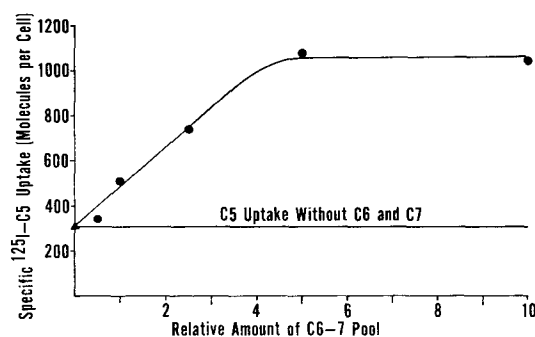

Fig. 12. Effect of C6 and C7 on the uptake of ^{125}I-C5. Increasing amounts of a pool of C6 and C7 were added to a mixture of ^{125}I-C5 and EAC1,4,^{oxy}2,3. After 60 min at 37°C, the cells were washed and C5 uptake was determined.

The Question of Cell-to-Cell Transfer of Activated C5.—Shin, Pickering, and Mayer (19) have shown that incubation of a mixture of EAC1,4,2,3 and EAC1,4,2,3,5 renders both cell types susceptible to lysis by C6-9. They have interpreted this as indicating cell-to-cell transfer of activated C5. The following experiments, however, make necessary a different interpretation of this phenomenon. EAC1,4,^{oxy}2,3,5 were added in varying numbers to 3 × 10^7 cells of different ^{51}Cr-labeled intermediate complexes. After incubation for 10 min at

30°C in a total volume of 50 μl of EDTA-GVB, C6-9 was added. In this experiment, EAC1,4,^{oxy}2,3,5 alone were 92% lysed. Upon admixture with ^{51}Cr-EAC1,4,^{oxy}2,3 (Fig. 13), however, there was an absolute increment in free hemoglobin due to lysis of ^{51}Cr-EAC1,4,^{oxy}2,3. Lysis and ^{51}Cr release were proportional to the number of EAC1,4,^{oxy}2,3,5 cells present. This effect was specific for EAC1,4,^{oxy}2,3 as recipient cells, as neither EAC1,4,^{oxy}2, EAC1,4 nor E were hemolysed. It should be emphasized that the amount of lysis of EAC1,4,^{oxy}2,3,5 obtained in these studies was unchanged in the presence of other cell types. Identical results were also obtained when EAC1,4,^{oxy}2,3,5,6, 7 were used as donor cells for C5.
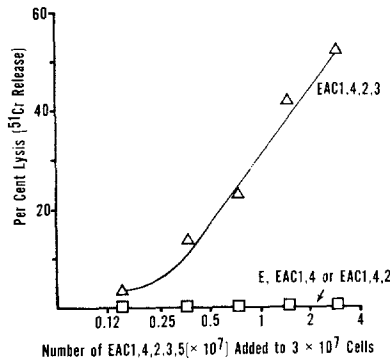

Fig. 13. Lysis of EAC1,4,^{oxy}2,3 in the presence of EAC1,4,^{oxy}2,3,5 and C6-C9. Varying numbers of EAC1,4,^{oxy}2,3,5 were added to 3 $\times$ 10^6 cells of different ^{51}Cr-labeled intermediate complexes, and after 10 min at 30°C, C6-C9 was added. EAC1,4,^{oxy}2,3 were lysed in proportion to the number of C5 cells present in the same reaction mixtures. The C5 cells were 92% lysed by C6-C9 whether tested alone or in the presence of other cell types.

The results of the above studies, in particular the observation that lysis of the donor cells was not reduced in the presence of recipient cells, tended to negate transfer of activated C5 as responsible for the lysis of EAC1,4,^{oxy}2,3. An alternative explanation was then considered, namely that this phenomenon is due to the utilization of nonspecifically adsorbed, native C5 and not to transfer of activated C5. This hypothesis was verified in the following experiments. Unsensitized red cells (E) were incubated with three amounts of C5 for 10 min at 30°C. These E + C5 cells were thoroughly washed and added to EAC1,4,^{oxy}2,3 in experiments identical to those described earlier. As shown in Fig. 14, the E + C5 cells were able to donate C5 to EAC1,4,^{oxy}2,3 and thus were able to completely duplicate the results obtained earlier with EAC1,4,^{oxy}2,3,5. The E + C5 cells were not lysed in this reaction, which was specific in that only EAC1,4,^{oxy}2,3 cells could serve as recipient cells and EAC1,4,^{oxy}2, EAC1,4, and E could not.

DISCUSSION

These experiments were initiated to clarify the mode of action of C5 in immune hemolysis. The studies presented here show that a small proportion of C5 offered to EAC1,4,2,3 becomes specifically cell bound (Figs. 2, 10) in a cytolytically active form (Fig. 11). It is thus clear that the fifth reaction step may proceed via the formation of a hemolytically active cellular intermediate complex, as proposed by the sequential action hypothesis (2). The extent of formation of the C5 intermediate complex was found to be controlled by cell-bound $\overline{C4,2}$ and C3 with each increment in the number of $\overline{C4,2}$ or C3 molecules per cell being reflected in greater binding of C5 (Fig. 3). Although only a minor pro-
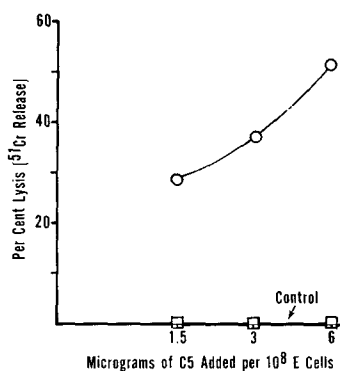

Fig. 14. Utilization of C5 adsorbed to E in lysis of EAC1,4,2,3 by C6-C9. Nonsensitized E were incubated with varying amounts of C5 (E + C5), washed, and added to ^{51}Cr-EAC1,4, ^{oxy}2,3 and other cellular intermediate complexes. C6-C9 was then added. The line shows the lysis of C3 cells obtained; neither C4 cells, C2 cells, nor E were lysed in mixtures with E + C5 (control).

portion of the C5 offered to EAC1,4,2,3 became physically cell bound, all of the C5 participated in the reaction as evidenced by its time-dependent loss of activity in the fluid phase. These data, along with the observation that C5 is cleaved by EAC4,2,3 (20), indicate that the C5 reaction step is mediated by a cell-bound complement enzyme, most likely $\overline{C4,2,3}$ (21). It is probable that the C5 intermediate is formed by a process of activation–transfer-binding identical to that postulated for the reaction steps involving C2 (5), C3 (14) and C4 (22).

The hemolytic activity of the C5 intermediate complex was found to be labile (Fig. 4), a feature which characterizes the C5 step as potentially rate-limiting in the complement reaction. While it was initially felt, in comparison with the C2 step, that the lability was probably secondary to dissociation of C5 from the cells, this hypothesis could not be verified. Experiments with radiolabeled C5 (Fig. 5) as well as neutralization-competition tests failed to show sufficient elu-

tion of C5 protein to explain the lability of bound C5 hemolytic activity. Nor could the lability be attributed to elution of ^{oxy}C2 from EAC1,4,^{oxy}2,3,5 (Fig. 6) or to a change in the functional status of bound C2 (Fig. 7). Therefore, the loss of activity of bound C5 must be a property of the C5 molecule itself. Since the majority of C5 molecules do not dissociate from cells, C5 must undergo a change *in situ* which renders it hemolytically unreactive. Although the nature of this alteration is unknown, it is not correlated with a major change in the size of the molecule (Fig. 8).

While the above studies show that the C5 reaction step may progress via the formation of an intermediate complex in a manner analogous to the reaction mechanism of the other complement components (1), the C5 step is unique in that it may also proceed via an alternative pathway. This alternate mechanism of C5 action was suggested by studies which showed that C5 uptake and hemolytic activity were markedly enhanced when the C5 reaction step was carried out in the presence of C6-C9 (Table I). Further study showed that isolated C6 and C7 greatly increased the uptake and, hence, hemolytic activity of C5 (Fig. 12, Table I). This alternative pathway, in which C5, C6, and C7 act together, probably explains the formation of EC5,6,7 which occurs when a mixture of unsensitized erythrocytes (E), C3, C5, C6, and C7 is treated with preformed $\overline{C4,2}$ (23). As shown in this laboratory (23 and footnote 2), neither a C5 nor C5,6 intermediate complex with E can be formed, the simultaneous presence of C5, C6, and C7 being necessary. Lachmann and Thompson, in studies of reactive hemolysis (24), have found that C5 and C6 form a complex after activation which is unable to bind to E unless C7 is supplied. Under these conditions, EC5,6,7 is formed. While the simultaneous requirement for all three components for binding may be related to the ability of these components to form a protein–protein complex (3), the underlying mechanism of the alternative pathway is unknown.

Despite the enhancement of C5 uptake produced by C6 and C7, the efficiency of binding of C5 remained low in comparison to that observed with other components (1). Even with cells bearing large numbers of $\overline{C4,2}$ and C3 molecules per cell, the uptake remained less than 4% of input (Table I). This relative inefficiency of binding was offset, however, by the exceptionally high hemolytic efficiency of bound C5. An average of 7 bound C5 molecules per cell were sufficient to produce 63% lysis (Table I), i.e., one effective molecule per cell. When determined with cells bearing large numbers of $\overline{C4,2}$ and C3 molecules per cell, the efficiency was even more striking, with only three molecules per cell being required (Table I). Since the data conform to the one-hit theory of immune hemolysis (17), the interpretation is that one of these bound molecules produces a single lesion which results in lysis. Thus, in contrast to the results obtained in studies of the hemolytic efficiency of bound C2 (5), C3 (1) and C4 (8), relatively few molecules of C5 appear to be specifically bound at sites on the cell surface which are resistant to complement injury.

The C5 molecule is unique among the complement proteins in that it is readily adsorbed in native form to various intermediate complexes (Fig. 5) as well as to unsensitized erythrocytes. This nonspecifically bound C5 remains firmly attached, although it may be specifically utilized as a source of native C5 by EAC1,4,2,3 (Fig. 14) in an ongoing complement reaction.

These studies document the existence of at least five functional forms of the C5 molecule. These include: (a) native C5 present in serum, (b) C5 which is found adsorbed in apparently native form to erythrocytes (Fig. 14), (c) bound active C5 which is responsible for the hemolytic activity of the C5 intermediate complex, (d) unbound, inactive C5 which has entered into reaction with EAC1,4,2,3 but failed to become bound, and (e) bound, inactive C5 which has lost its hemolytic activity while remaining firmly attached.

SUMMARY

The data presented here indicate that the C5 reaction step may proceed via the specific attachment of C5 to EAC1,4,2,3 and the formation of a hemolytically active C5 intermediate complex. During this process only a minor proportion (less than 4%) of C5 offered to EAC1,4,2,3 becomes bound, although the remaining C5 also participates in the reaction as evidenced by its inactivation in the fluid phase. Once bound, C5 is exceptionally efficient in producing hemolysis, requiring less than seven specifically bound molecules per cell for the production of a hemolytic lesion. The extent of formation of the C5 intermediate complex is primarily dependent on the number of molecules of $\overline{C4,2}$ and C3 present on the cells employed for its generation. In these respects, the mode of action of C5 is completely analogous to that of the other components of complement thus far investigated. The C5 step differs, however, in other aspects. The binding of C5 is influenced by C6 and C7, components which are thought to act subsequent to it in the complement sequence. In addition, the hemolytic activity of the isolated C5 intermediate complex is exceedingly labile, having an average half-life at 30°C of only 9 min. This characteristic distinguishes the C5 step, along with the C2 step, as potentially rate-limiting in the complement reaction. However, unlike C2, C5 remains firmly cell-bound during the decay process and apparently undergoes an alteration *in situ* which renders it hemolytically unreactive. Finally, C5 is unique in that it readily adsorbs in native form to unsensitized erythrocytes. This nonspecifically bound C5 remains firmly attached, although it may be specifically utilized as a source of C5 by an ongoing complement reaction. The significance of the marked affinity of native C5 for cell-surface receptors remains to be determined.

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