THE MEMBRANE ATTACK MECHANISM OF COMPLEMENT

Reversible Interactions among the Five Native Components in Free Solution*

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The present study was designed to investigate possible interactions among human C5, C6, C7, C8, and C9 in cell-free solution.

Previous work suggested that these components enter into close steric contact with each other when bound to the surface of a cell under attack by complement (1). Whereas binding of C5 required enzymatic activation, binding of C6, C7, C8, and C9 did not, and probably proceeded by adsorption. These findings suggested that the five proteins of the membrane attack mechanism might exhibit, in their native form, stereochemical affinity for each other.

In the following, the results of a series of ultracentrifugal experiments will be reported that show that a limited number of interactions can be observed when the five proteins are examined in various combinations. The fact that the interactions among the five proteins are limited indicates their specificity and suggests the existence of distinct topological relationships among the subcomponents of the recently recognized, stable C5–9 complex (2).

Materials and Methods

Human Complement Components and Erythrocyte-Antibody-Complement Complexes.—Details have been outlined in the accompanying paper (2).

Hemolytic Assays of Individual Components.—Samples were assayed for the following hemolytic activities by incubation with 3×10^7 sheep erythrocyte[1]-complement interme-

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[1] *Abbreviations used in this paper:* E, sheep erythrocytes; EA, antibody-sensitized sheep erythrocytes; GVB, VB-NaCl containing 1.0% gelatin; GVBE, GVB containing 5 mM EDTA; HSA, human serum albumin; IgG, 7S gamma globulin; VB-NaCl, Veronal-buffered (5 mM) saline containing 1.5×10^{-4} M $CaCl_2$, 5×10^{-4} M $MgCl_2$.

diate complexes and reagents in a final volume of 500 μl. C3: EAC1, 4, $^{oxy}2$ + 25 μl hydrazine-KSCN serum (3) (containing 5 mM EDTA) + 0.1 μg C5; C5: EAC1-3 + 25 μl hydrazine-KSCN serum (containing 5 mM EDTA); C6: EAC1-3 + 25 μl C6-deficient rabbit serum; C7: EAC1-3 + 0.1 μg C5 + 0.6 μg C6 + 0.005 μg C8 + 0.1 μg C9; C8: EAC1-7 + 0.1 μg C9; C9: EAC1-7 + 0.05 μg C8. GVBE was employed as diluent for the C3 and C5 assays and GVB for the C6, C7, C8, and C9 assays. After incubation at 37°C for a period of time sufficient to give maximally 80% lysis, 1 ml of ice-cold isotonic sodium chloride was added to each tube. The unlysed cells were removed at 900 *g* in a Sorvall model GLC-1 (Ivan Sorvall, Inc., Norwalk, Conn.) for 10 min and the amount of oxyhemoglobin released was quantitated at 412 nm.

Sucrose Density Gradient Ultracentrifugation.—7.7–31% linear sucrose gradients in Veronal-buffered sodium chloride, pH 7.4, ionic strength 0.05, were formed in 5-ml cellulose nitrate tubes by using a Buchler automatic density gradient maker (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N. J.). The gradients were equilibrated to 4°C before the samples (200 μl) were applied. Ultracentrifugation was performed in an SW 50 or SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 15 h at 39,000–45,000 rpm and 4°C. Gradients of various ionic strength were made by dissolving the sucrose in 5 mM Veronal buffer, pH 7.5, containing the indicated amount of sodium chloride as the electrolyte. Fractions were collected by puncturing the bottom of the tubes. [^{131}I]IgG (7S) and [^{131}I]HSA (4.5S) were added as markers to the protein mixtures applied to the gradients. Radiolabeling was performed according to McConahey and Dixon (4).

Evaluation of Positive and Negative Reactions.—Interaction among different proteins was considered positive when a reproducible increment in sedimentation coefficient of one or more components was observed in the test mixture, as compared with individual controls under otherwise identical conditions. A reaction was judged negative when the sedimentation behavior of all components in the test mixture was identical with that of the individual controls.

RESULTS

Demonstration of Reversible Association between C8 and C9.—In all experiments reported below the method chosen for demonstration of reversible protein-protein interactions among the five isolated complement components studied was sucrose density gradient ultracentrifugation.

Association between C8 and C9 in free solution is shown in Fig. 1. In the presence of C8, C9 sedimented with an *s* rate of 10.2S instead of 4.8S, which is characteristic for C9 examined in the absence of C8. C8 in mixture with C9 exhibited a bimodal distribution, the rapid portion sedimenting together with C9 (10.2S) and the slow portion having an *s* rate of 8.5S, which is characteristic for C8 examined by itself. The conditions chosen in this experiment were: pH 7.5, ionic strength 0.05, and a molar ratio of C8 to C9 of 2.5 to 1 (50 μg of C8 and 10 μg of C9). Since both C8 and C9 had an accelerated velocity, as evidenced by the appearance of the 10.2S component, it is concluded that both proteins entered, under the conditions employed, into a relatively stable association product (C8, 9).

The intensity of many protein-protein interactions is inversely related to the ionic strength of the medium in which they are observed. Fig. 2 shows the dependence of C8, 9 association upon ionic strength. At $\mu = 0.05$ the distribution of the two components was as described above. At $\mu = 0.1$, C9

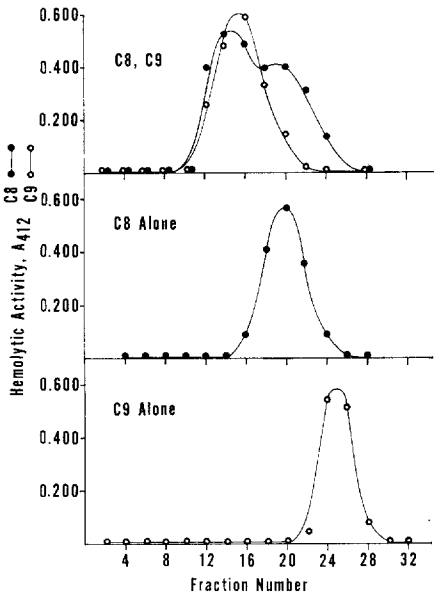

FIG. 1. Interaction between native C8 and C9 in free solution as demonstrated by sucrose density gradient ultracentrifugation. 10 μg of C9 and 50 μg of C8, individually or as a mixture, were placed onto a 7.7-31% linear sucrose gradient in VB-NaCl, pH 7.5, ionic strength 0.05, and centrifuged for 15 h at 39,000 rpm at 4°C. The direction of sedimentation was from right to left.

was partially associated with C8 and partially free. At μ = 0.15 the major portion of C9 sedimented independently of C8. The dependence of C8, 9 interaction on ionic strength is consistent with its reversibility.

In order to demonstrate directly the reversibility of this association, the C8, 9 complex observed at μ = 0.05 (Fig. 2, *upper panel*) was reexamined by ultracentrifugation at the same and at a higher ionic strength. Whereas at μ = 0.05 it retained the sedimentation behavior of the complex, it completely dissociated at μ = 0.15 (Fig. 3).

The effect of pH was studied over a range of 6.5–8.5 at constant ionic strength (μ = 0.05). The results, which are not shown, indicated that association was independent of pH over the range studied.

The effect of relative protein concentration at μ = 0.05 is shown in Fig. 4. In these experiments the amount of C9 was constant and the amount of C8 was varied. When C9 was in excess (molar ratio C8:C9 = 1:2), the major portion of C9 remained apparently free. When C8 was in excess (molar ratio C8:C9 = 2.5:1), all of the C9 present sedimented in association with C8.

Differential Affinity of C8 and C9 for C5, 6, 7.—Fig. 5 shows the sedimentation behavior of C5, C6, and C7 when subjected to ultracentrifugation individually. Fig. 6, *upper panel*, demonstrates the previously described interaction

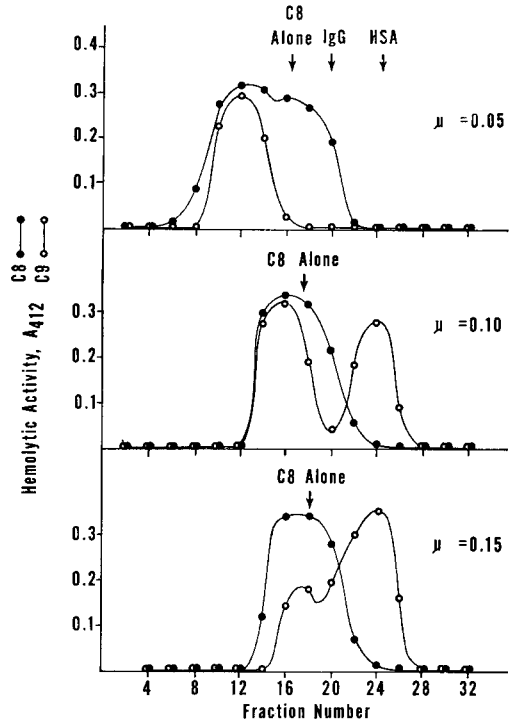

FIG. 2. C8, 9 association in free solution as a function of ionic strength. 50 μg of C8 and 10 μg of C9 were mixed at the indicated ionic strength. The samples were subjected to sucrose density gradient ultracentrifugation in VB-NaCl, pH 7.5, and indicated ionic strength.

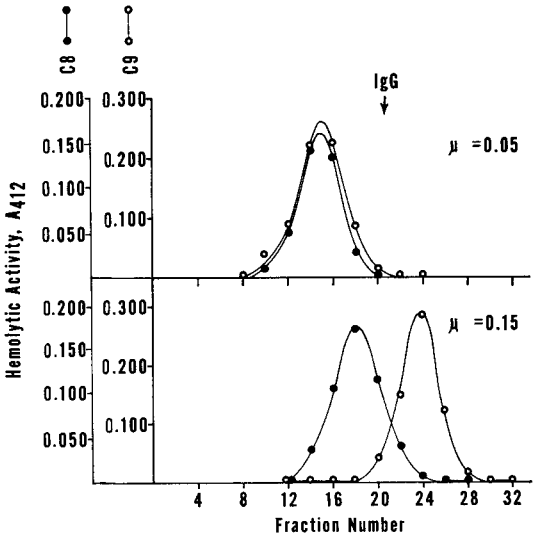

FIG. 3. Demonstration of reversibility of the C8, 9 association on raising the ionic strength. C8, 9 complex obtained from fractions of a zone ultracentrifugation experiment using μ = 0.05 (Fig. 2, *upper panel*) was reexamined by ultracentrifugation at μ = 0.05 (*top panel*) and μ = 0.15 (*bottom panel*).

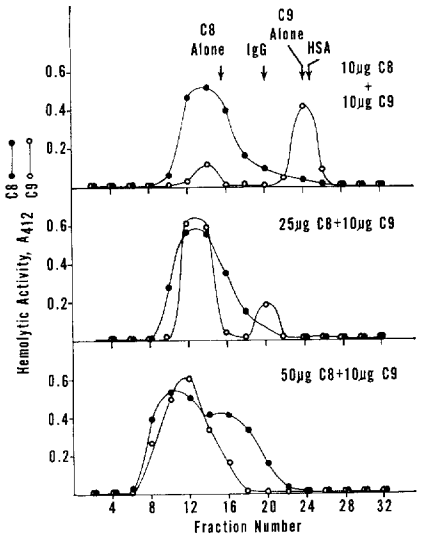

FIG. 4. Association of C8 and C9 as a function of relative protein concentration. A constant amount of C9 (10 μg) was mixed with increasing amounts of C8 (10, 25, and 50 μg) before analysis by ultracentrifugation. The sucrose density gradients employed were as described in the legend to Fig. 1.

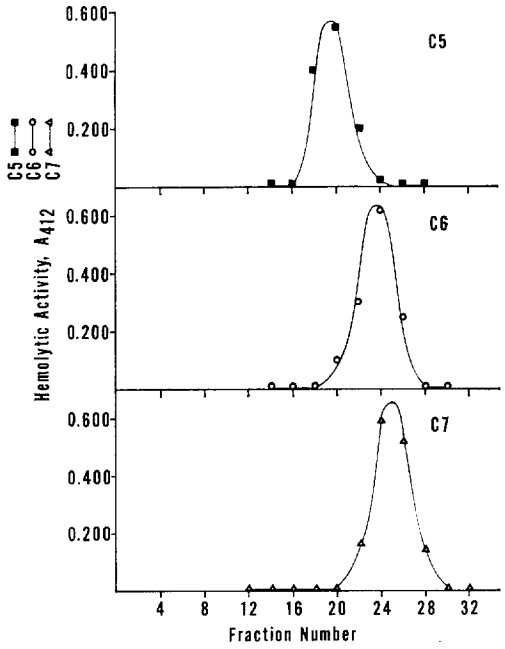

FIG. 5. Sedimentation behavior of C5, C6, and C7 analyzed individually by sucrose density gradient ultracentrifugation. Conditions as described in the legend of Fig. 1.

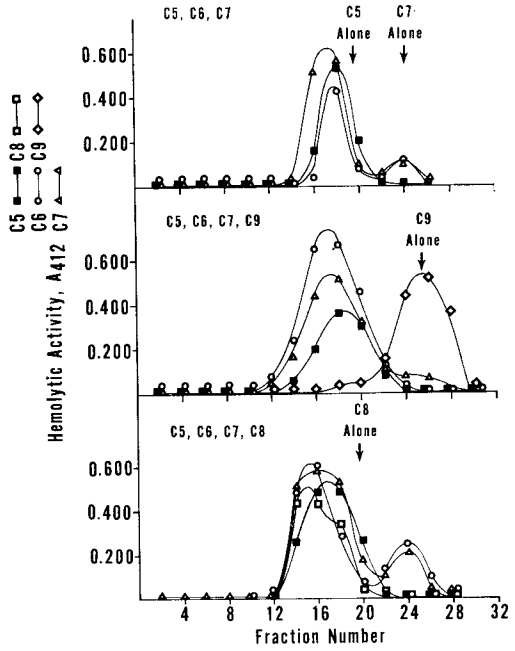

FIG. 6. Differential affinity of C8 and C9 for C5, 6, 7. C5, C6, and C7 (*upper panel*) were mixed with C8 (*bottom panel*) or C9 (*middle panel*) before the mixtures were subjected to sucrose density gradient ultracentrifugation as described in the legend to Fig. 1. The following amounts were employed: C5, 85 μg; C6, C7, and C9, 10 μg; C8, 50 μg.

of C5, C6, and C7 (5). The major portions of C6 and C7 sediment in association with C5 at a velocity of 9.2S. Addition of C9 to this mixture had no effect on the *s* rate of the three proteins nor had the C5, 6, 7 complex any effect on the *s* rate of C9. Addition of C8 to C5, C6, and C7 reproducibly accelerated the velocity of C8 from 8.5S to 9.6S, so that all four proteins were found in the same zone. In addition, evidence was obtained for a direct interaction of C5 and C8 in absence of C6 and C7. However, no demonstrable reaction occurred between either C6 and C8 or C7 and C8.

That the described reactions have a high degree of specificity is indicated by the data listed in Tables I and II. According to these data, C8 appears unable to associate with either C3 or human albumin; and C9 failed to associate with either C5 alone, C5, 6, 7, or IgG.

Demonstration of an Association Product of Native C5, C6, C7, C8, and C9.— Fig. 7 depicts the result obtained when a mixture of the five components of the membrane attack mechanism was subjected to ultracentrifugation. In spite of the limited number of different affinities existing between these proteins, association of all five components did occur. The association product sedimented within an *s* rate range of 10.8–11.2S. In addition, small amounts of

TABLE I

Specificity of Fluid Phase Interactions between C8 and C9 as Shown by Sucrose Density Gradient Ultracentrifugation

Proteins applied	Amounts used	Maximal sedimentation coefficient of C9*
C9	10 μg	4.8
IgG + C9	50 μg + 10 μg	4.8
C5 + C9	85 μg + 10 μg	4.8
	10 μg + 50 μg	4.8
C5 + C6 + C7 + C9	85 + 10 + 10 + 10 μg	4.8
C8 + C9	10 μg + 10 μg	7.7
	25 μg + 10 μg	8.3
	50 μg + 10 μg	10.6

* Conditions: VB-NaCl, pH 7.5, $\mu = 0.05$; 7.7–31% sucrose density gradient; 16 h, 39,000 rpm.

TABLE II

Specificity of Fluid Phase Interactions between C5 and C8 as Shown by Sucrose Density Gradient Ultracentrifugation

Proteins applied	Amounts used	Maximal sedimentation coefficient of C8*
C8	50 μg	8.5
C8 + HSA	50 μg + 100 μg	8.5
C8 + C3	100 μg + 10 μg	8.5
C8 + C5	50 μg + 50 μg	9.5
C5 + C6 + C7 + C8	85 + 10 + 10 + 50 μg	9.5

* Conditions: see Table I.

unassociated C6, C7, and C9 were observed. Although the quantities of C8 and C9 employed, as well as the experimental conditions, were identical with those used earlier for experiments performed with a mixture of C8 and C9 alone (Figs. 1 and 2), the relative distribution of the two proteins was different. Obviously the availability of binding sites for C9 on C8 was reduced in the presence of C5, 6, 7. Virtually identical results were obtained when whole human serum was subjected to ultracentrifugation under identical conditions.

DISCUSSION

Since the interaction studies were performed with isolated proteins, the question arises as to whether the ability to interact is a property of the native proteins or whether it was acquired during the purification process. In order

TABLE III

Observed Interactions among Native C5–9

C5 + C6 ⇌ C5, 6
C5 + C7 ⇌ C5, 7
C5 + C6 + C7 ⇌ C5, 6, 7
C5 + C8 ⇌ C5, 8
C5 + C6 + C7 + C8 ⇌ C5, 6, 7, 8
C8 + C9 ⇌ C8, 9
C5 + C6 + C7 + C8 + C9 ⇌ C5, 6, 7, 8, 9

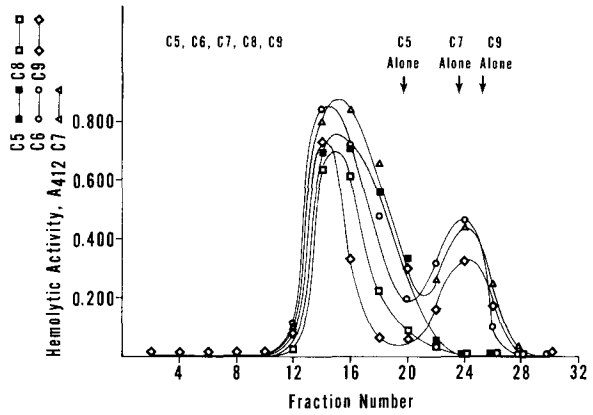

FIG. 7. Demonstration of a pentamolecular association product of native C5, C6, C7, C8, and C9. 85 μg of C5, 10 μg each of C6, C7, and C9, and 50 μg of C8 were mixed together and subjected to zone ultracentrifugation as described in the legend to Fig. 1.

to evaluate the potential biologic significance of the observed interactions, it was necessary to know whether they occur in whole serum in spite of the presence of many other proteins. The observations made on whole human, rabbit, and guinea pig serum were essentially identical with those with isolated components. It may be concluded therefore that the C5–9 interactions are a function of unaltered, native complement proteins, that they are highly specific, and not inhibited by the bulk of other serum proteins.

Interactions similar to those reported in this paper were previously observed with native, isolated C2 and C4 (6). The functional relevance of this reaction is evident from the fact that on enzymatic activation these two proteins combine to form the bimolecular enzyme C3 convertase (6). By analogy the delineated affinities exhibited by the native C5–9 proteins may indicate the manner in which they are organized into a cytolytically active complex. A C5–9 complex with direct membrane damaging activity was previously postulated on the basis of work with cell-antibody-complement complexes (1). It was envisioned to contain a trimolecular arrangement of C5b, C6, and C7,

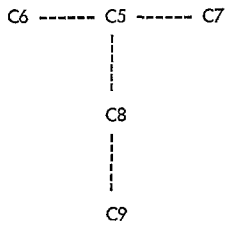

FIG. 8. Schematic representation of verified interactions between C5–9, showing the manner in which these five complement proteins in their native form can enter into association in free solution.

which provides the binding site for one C8 molecule. The resulting tetramolecular complex was assigned the geometry of a tetrahedron. The bound C8 molecule was considered to furnish binding sites for six C9 molecules. The present observations regarding the existence of stereochemical affinities among this group of proteins is consistent with the previously advanced concept. As summarized in Table III and Fig. 8, the essential interactions occur between C5, C6, and C7, between C5 and C8, and between C8 and C9. This set of interactions accounts for the ability of the five proteins to enter in association with each other and may reflect essential structural features of both the cell-bound and the soluble C5–9 complex (2).

SUMMARY

Reversible interactions in free solution were demonstrated to occur (*a*) between C5 and C8, (*b*) between C5, 6, 7 and C8, and (*c*) between C8 and C9. No interaction was observed between C8 and C6 or C7 and between C9 and C5, 6, 7. Interactions between C8 and C9 were enhanced at lowered ionic strength (0.05) and a molar excess of C8 over C9. Complex formation was independent of pH over the range of 6.5–8.5. Under optimal conditions the C8, 9 complex had a sedimentation coefficient of 10.2–10.6S, while native C8 and C9 sedimented at 8.5 and 4.8S, respectively. Specificity and reversibility of these interactions were established.

In spite of the limited number of interactions observed, all five of the native proteins of the membrane attack mechanism interacted to form an association product that sedimented at 10.8–11.2S. Demonstration of this product in free solution supports the concept that C5–9 on acquisition of cytolytic activity assemble into a stable multimolecular complex.

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