FORMATION AND FUNCTIONAL SIGNIFICANCE OF A MOLECULAR COMPLEX DERIVED FROM THE SECOND AND THE FOURTH COMPONENT OF HUMAN COMPLEMENT*, ‡

By HANS J. MÜLLER-EBERHARD, M.D., MARGARET J. POLLEY, PH.D., AND MARY ANN CALCOTT

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California)

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Studies on the mechanism of immune cytolysis have been greatly facilitated by the use of highly purified complement components. Paradoxically much can be learned about immune cytolysis by studying the reactions of complement components in solutions which are entirely devoid of cells. The experiments described in the present paper were performed to explore the molecular interactions between the first four components of human complement in cell- and antibody-free solution. Use was made of highly purified preparations of the esterase moiety of the first component (C'1), and of the second (C'2), third (C'3), and fourth (C'4) component. It was found that C'2 and C'4 tend to interact in free solution to form a reversible protein-protein complex which is converted into a stable complex by the action of C'1 esterase. Formation of the esterase-induced complex results in the generation of an enzyme-like activity which causes the conversion of C'3 to its hemolytically inactive product, C'3i (1). Evidence will be presented indicating that the complex is formed not only in cell-free solution, but also on the surface of cells and that it fulfills an essential function in cell membrane damage by complement.

Materials and Methods

Purified Human Complement Factors.—The esterase moiety of the first component of complement (2) was isolated and kindly provided by Dr. Irwin Lepow, Cleveland, Ohio.

The second component was purified from the hydrazine-treated pseudoglobulin fraction of human serum by a three step procedure which will be described in detail elsewhere.[1] Briefly, the first step consists of chromatography on carboxymethyl-cellulose using phosphate buffer, pH 6.0, ionic strength 0.05 containing 0.0025 M EDTA. The protein is eluted by an increasing

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sodium chloride concentration gradient. In the second step the portion of the chromatogram which contains C'2 activity is further separated by preparative electrophoresis on a Pevikon block using the same buffer as above. At the conclusion of this step, C'2 is approximately 5000-fold purified and remains contaminated primarily with β_{1A}-globulin. As revealed by starch gel electrophoresis, C'2 represents approximately 30% of the protein of this material. Most experiments were carried out with "post-Pevikon block C'2." In a few instances the product of the third and final purification step was used which involves either chromatography on TEAE-cellulose or Sephadex filtration.

The third and the fourth components of complement were prepared according to standard procedures which were described previously (3, 4).

Chemically and Enzymatically Modified Complement Components.—Oxidation of C'2 was carried out by treating the purified component at a protein concentration not exceeding 1 mg/ml with an equal volume of 10^{-4} M I_2 in 5×10^{-8} M KI at pH 6 and 0°C for 5 min. Oxidized C'2 is 7–10 times as active as untreated human C'2. The method of oxidation has been described briefly (5); the evidence that the enhancing effect of iodine is due to oxidation and not due to iodination will be presented elsewhere.[1]

C'3 was labeled with radioactive ^{131}I or ^{125}I using the chloramine T method, as described earlier (1, 6). The hemolytic activity of the labeled protein was unimpaired, its specific radioactivity was approximately 50,000 cpm/μg.

To obtain C'4i (monomer), C'4 was treated with C'1 esterase as outlined previously (7). The C'1 esterase treated, hemolytically inactive C'4 was then subjected to sucrose density gradient ultracentrifugation to separate C'4i-aggregates (12S-14S) from C'4i monomer (8.9S). Alternatively, the monomeric form of C'4i was isolated by gel filtration using Sephadex G-200 equilibrated with phosphate buffer, pH 6.0, ionic strength 0.15. The aggregates appear in the exclusion volume, while the monomer is retarded. Fractions containing the monomer were pooled, dialyzed against veronal buffer, pH 7.3, and concentrated by ultrafiltration to a protein concentration of 1–4 mg/ml.

Detection and Quantitation of C 3 Conversion.—Conversion of C'3 to C'3i is readily detected by immunoelectrophoresis. It was quantitated using radioactively labeled C'3. Conversion was stopped by cooling the reaction mixtures to 0°C. Subsequent separation of C'3 and C'3i and quantitative evaluation were described earlier (1).

Detection and Estimation of C'2 Activity.—This was done either using the classical serum reagent, R2 (8), or utilizing the conversion of EAC'1a,4 cells to EAC'1a,4,2a by C'2. The former were prepared as described earlier (1) and the latter were assayed using EDTA serum (8).

Preparation of C'3-Converting Activity in Cell- and Antibody-Free Solution: (C'4i,2)a.—20 μg of C'2 or of iodine-treated C'2 was mixed with 200 μg of C'4. Usually phosphate buffer of pH 6.5–7.5 and ionic strength 0.15 was used which contained 1.5 mM Mg^{++}. The total reaction volume was 0.5 ml. After the temperature was adjusted to 32°C, 0.5 units of purified C'1 esterase was added. The reaction was allowed to proceed for 10 min at 32°C when the complex was made with iodine-treated C'2 and for 2 min when untreated C'2 was employed. The material was then utilized immediately or stored at 0–2°C. (C'4i, 2)a made with iodine-treated C'2 retained C'3 converting activity for several days when stored in the refrigerator.

Preparation of Sheep-Erythrocyte-Complement Complexes.—EC'4, the erythrocyte-C'4 complex, was prepared as described previously (7).

EC'4, ^{oxy}2a, erythrocytes carrying on their surface only the activated complex of C'4 and oxidized C'2, were prepared in the following manner. 2 ml of a 7.5% suspension of EC'4 in veronal buffer, pH 7.3, containing Ca^{++} and Mg^{++} was placed at 32°C. 0.4 ml of purified, iodine-treated C'2 was added which was followed after 2 min by addition of 0.25 units of purified C'1 esterase. The total reaction volume was 2.5 ml, the time of incubation at 32°C

was 20 min. The cells were washed twice with ice-cold veronal buffer, their concentration adjusted to 2.5%, and they were then stored at 2°C until used. They were lyzed by incubation at 32°C with human serum, 1:40, in the presence of 0.01 M Na_3EDTA.

Sucrose Density Gradient Ultracentrifugation.—Linear sucrose density gradients (7–31%) were produced with the Buchler automatic density gradient device. Sucrose solutions were made up in phosphate buffer of ionic strength and pH indicated below. Ultracentrifugation was performed for 20 hr at 35,000 rpm and 4°C in a Spinco L-2 machine employing an SW39 rotor. Fractions were obtained using the Buchler gradient fractionation device. For *s* rate determinations the following reference substances were used: ^{125}I-labeled thyroglobulin (19S), ^{125}I-labeled γG-globulin (7S), and hemoglobin (4.5S).

Gel Filtration.—A 100 × 2 cm column, Sephadex G-200 and phosphate buffer, pH 6.0, ionic strength 0.15 were used. The length of the gel bed was 80 cm. For application the density of the sample was raised by addition of NaCl and the sample was layered between the gel bed and the supernatant buffer. 1–2 ml fractions were collected. For the determination of diffusion coefficients, the above reference substances were employed, thyroglobulin (2.5×10^{-7} cm^2/sec), γG-globulin (3.8×10^{-7} cm^2/sec), and hemoglobin (6.8×10^{-7} cm^2/sec). The diffusion coefficient of C'2 was also determined in veronal buffer, pH 7.4, ionic strength 0.15, containing 0.005 M Na_2EDTA.

Quantitation of Formation and Decay of (C'4i,2)a.—Two different reaction mixtures were prepared, both containing 80 μg of C'4, 1.5 mM Mg^{++} and 0.05 units C'1 esterase in isotonic veronal–NaCl buffer, pH 7.3. One contained, in addition, 20 μg of iodine-treated C'2 and the other 100 μg of untreated C'2. C'1 esterase was added last after the temperature of the solutions had been adjusted to 35°C. The total reaction volume was 0.5 ml and 0.05 ml samples were withdrawn at indicated intervals of time after the reaction was triggered by addition of C'1 esterase. The samples were pipetted into ice-cold tubes containing 10 μl of 0.1 M EDTA. After all samples were collected, they were assayed for C'3-converting activity as described above.

Nomenclature.—The complement nomenclature used in this publication conforms with the recommendations of the active members of the Complement Workshops held in Bethesda, 1963 (9) and in La Jolla, 1966 (10). Individual complement components are designated numerically. Erythrocyte (E)-antibody (A)-complement (C') complexes are denoted systematically according to their composition or according to the reaction step they have completed; e.g., EAC'1, EAC'1,4, EAC'1,4,2, etc. Since C'1 and C'2 have to undergo activation (a) before they become hemolytically effective, these complexes are more accurately referred to as EAC'1a, EAC'1a,4, EAC'1a,4,2a. After inactivation by C'1 esterase C'2 is called C'2i (11).

To convey the results of recent studies undertaken in this laboratory, the following additional symbols and terms will be used in the present and in subsequent publications. They are constructed in accordance with the general rules for complement nomenclature.

C'4i: C'1 esterase-treated C'4 which has undergone characteristic physicochemical changes and has lost the capacity to become bound to groups which serve as C'4 receptors.

C'3i: C'3 convertase (see below)-treated C'3 which has undergone characteristic chemical changes and which has lost the capacity to become bound to groups which serve as C'3 receptors.

^{oxy}*C'2:* C'2 oxidized by treatment with a critical concentration of iodine, a measure which results in a 10-fold enhancement of its hemolytic activity.

C'4,2: Reversible protein-protein complex of hemolytically active C'2 and C'4; complex is devoid of C'3 convertase activity.

C'4i,2: Reversible protein-protein complex of C'4i and C'2; devoid of C'3 convertase activity.

(C'4i,2)a: Protein-protein complex, derived from C'4 and C'2 by the action of C'1 esterase

in the presence of Mg^{++}; possessing capacity to convert C'3 to C'3i and to induce hemolysis from the fluid phase with low efficiency; once formed the complex is incapable of becoming bound to receptor groups owing to the effect of C'1 esterase on its C'4-derived subunit.

C'4,2a: Receptor-bound complex possessing C'3-converting activity; exhibiting high cytolytic efficiency if bound to cell surface receptors.

C'3 convertase: Trivial name for the fluid phase complex, (C'4i,2)a, and the receptor-bound complex, C'4,2a; referring to its enzymatic effect on C'3.

RESULTS

Which Complement Components are Required for the Generation of C'3-Converting Activity in Cell-Free Solution? As described previously (1), conversion of C'3 to C'3i is accompanied by loss of hemolytic activity and by characteristic

TABLE I

Generation of C'3-Converting Activity in Free Solution

Components	C'3-converting activity
C'1 esterase	Negative
C'1 esterase, C'4	Negative
C'1 esterase, C'2	Negative
C'1 esterase, C'4, C'2	Positive
C'1 esterase, C'4 (30', 37°C), C'2	Positive
C'1 esterase, C'2 (30', 37°C), C'4	Negative

Conditions: C'1 esterase, 0.5 unit; C'2, approximately 1 μg; C'4, 1 μg; C'3, 180 μg; total volume, 0.15 ml; time at 37°C, 20 min.

physicochemical and immunochemical changes of this protein. Conversion is a necessary part of the mechanism of binding of C'3 to cells and is mediated only by those cells which contain the activated second component on their surface. The kinetics and the conditions of the conversion have suggested that it is an enzymatic reaction.

To study the manner in which C'3-converting activity is generated and to identify its physicochemical correlate, the following experiments were carried out. Small amounts of highly purified C'1 esterase (0.5 unit), C'2 (approximately 1 μg), and C'4 (1 μg) were incubated in various combinations with 180 μg of C'3 in Mg^{++}-containing buffer. After 20 min at 37°C the reaction mixtures were analyzed for C'3i. The results are summarized in Table I. C'3-converting activity was generated neither when C'2 nor C'4 were treated with C'1 esterase. It was generated, however, when a solution containing both C'2 and C'4 was subjected to the action of the enzyme. The activity also appeared when C'4 was first incubated alone with C'1 esterase and when C'2 was added to this mixture 30 min later. When the sequence of addition was

reversed, i.e. when incubation of C'2 with the enzyme preceded addition of C'4, C'3-converting activity failed to be generated. Furthermore, formation of the activity was inhibited by 0.01 M EDTA. This effect could be overcome by addition of Mg ions.

These observations indicated that both C'2 and C'4 were required for induction of C'3-converting activity. They further indicated that C'2 was inactivated when treated with the esterase in the absence of C'4,whereas C'4 retained its activity in the free solution system after treatment with esterase in the absence of C'2. It was postulated, therefore, that C'4 serves as an acceptor for

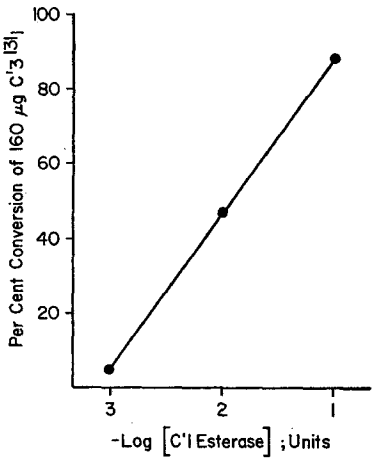

FIG. 1. Quantitative relation between the degree of conversion of C'3 and the amount of C'1 esterase present in the reaction mixture. The reaction was carried out for 30 min at 37°C in veronal buffer, pH 7.4, ionic strength 0.15. The total volume was 0.2 ml and contained 0.5 μg ^{oxy}C'2, 5 μg C'4, and 1.5 mM Mg^{++}.

esterase-modified C'2 and that C'3-converting activity resides in an esterase induced complex of C'2 and C'4.

Quantitative Relation Between C'3-Converting Activity and the Complement Components Required for its Generation.—To gain insight into the mode of action of C'3-converting activity, conversion was studied as a function of the concentration of C'1 esterase, C'2, and C'4. In these experiments two of the components were supplied in optimal concentration while the amount of the third was varied. Each reaction mixture contained 160 μg of C'3-^{131}I and the reaction was allowed to proceed for 30 min at 37°C, whereupon the amount of C'3i-^{131}I produced during the incubation period was determined. The results are demonstrated in Figs. 1–3, which show the relation between the degree of conversion and the concentrations of C'1 esterase, C'2, and C'4, respectively. The concentration of C'2 was recorded in relative values since the preparation

used was contaminated with β_{1A}-globulin, C'2 representing approximately one-third of the protein content of this material. On the basis of these determinations the efficiency of the three components could be calculated. It was found that, in the presence of optimal amounts of the other two components

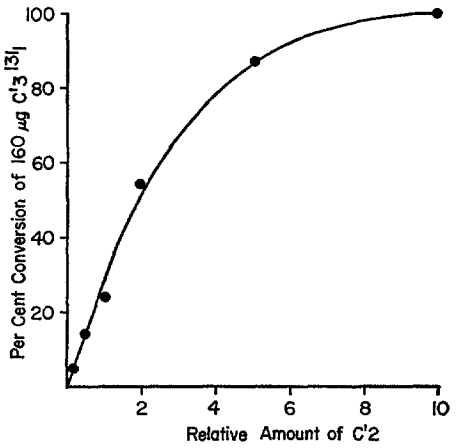

FIG. 2. Quantitative relation between C'3 conversion and the relative amount of C'2 in the reaction mixture. The reaction was carried out for 30 min at 37°C in veronal buffer, pH 7.4, ionic strength 0.15, containing 1.5 mM Mg^{++}, 0.5 unit C'1 esterase and 5 μg C'4. The total volume was 0.2 ml. In absolute terms the concentration of C'2 ranged approximately from 0.025 to 0.25 μg protein. C'2 was iodine treated.

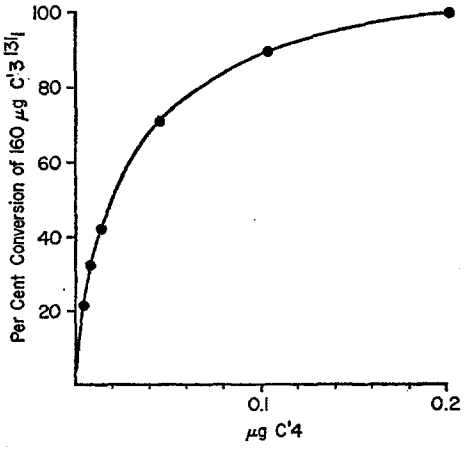

FIG. 3. Quantitative relation between C'3 conversion and the amount of C'4 in the reaction mixture. The reaction was carried out for 30 min at 37°C in veronal buffer, pH 7.4, ionic strength 0.15, and in the presence of 0.5 unit C'1 esterase, 0.5 μg ^{oxy}C'2 and 1.5 mM Mg^{++}. The total volume was 0.2 ml.

and under the selected experimental conditions, 10,000 μg of C'3 can be converted by 1.25 units of C'1 esterase, 12.5 μg of C'2, and 2.5 μg of C'4. The quantitative relations indicate that these components function as a catalyst in the conversion reaction. The trivial name C'3 convertase will be used in the following to denote the active principle.

Comparative Ultracentrifugal and Gel Filtration Studies of C'3 Convertase and its Precursors.—Experiments described above had suggested that C'3 convertase might constitute a protein-protein complex which is derived from C'2 and C'4. An ultracentrifugal analysis was therefore performed to compare the sedimentation velocity of C'3 convertase with that of its precursor components.

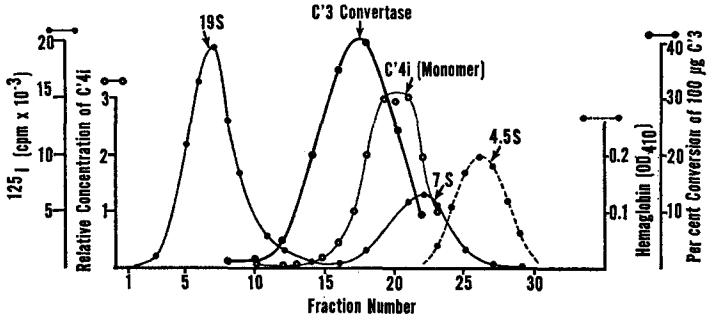

FIG. 4. Comparative ultracentrifugal analysis of C'3 convertase and one of its precursors, C'4i; performed in a 7–31% sucrose density gradient in phosphate buffer, pH 6.0, ionic strength 0.15; for 20 hr at 35,000 rpm As reference substances were used, 19S: thyroglobulin; 7S: γG-globulin; and 4.5S: hemoglobin.

The convertase was prepared from the monomeric form of highly purified C'4i and from partially purified C'2 by treatment with C'1 esterase in the presence of Mg^{++}. Usually the mixture was held for 5 min at 37°C before it was rapidly cooled to 1°C and subjected to ultracentrifugation in a sucrose density gradient. All initial attempts to recover C'3 convertase activity from gradient fractions failed. As will be seen below, the activity is exceedingly labile at 35°C. Although ultracentrifugation was carried out at 3–5°C, the failure to recover activity could be attributed to its decay during the 20 hr period of centrifugation.

Recovery difficulties were overcome when it was found that prior oxidation of C'2 with a critical dose of iodine (5) resulted in a much greater stability of the convertase. Accordingly, in all subsequent ultracentrifuge experiments C'2 was employed in its oxidized form. Fig. 4 depicts the result of a typical experiment and shows that C'3 convertase activity sediments more rapidly than C'4i, which in other experiments was found to be heavier than C'2 (see below). Using three reference proteins of known *s* rates, the sedimentation

velocities of C'3 convertase, C'4i, and C'2 were determined and found to be 11.2S, 8.9S, and 5.5S, respectively (Fig. 5). The former two values were arrived at by duplicate determination, the latter represents the mean of ten separate determinations.

The monomeric form of C'4i was used in these experiments in preference to native C'4. When C'4 is converted by C'1 esterase to C'4i, 30–50% of the protein forms aggregates which have an *s* rate of 12S–14S (7). By working with preformed C'4i monomer, it was possible to rule out that the aggregates were responsible for the sedimentation behavior of C'3 convertase.

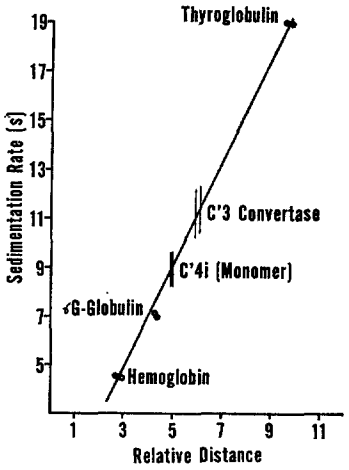

FIG. 5. Determination of the sedimentation coefficients of C'3 convertase and of C'4i after ultracentrifugation in a linear sucrose density gradient in the presence of three reference substances. The data were derived from two separate experiments.

To obtain approximate values of the diffusion coefficients (D) of C'3 convertase and its precursors, the method of Andrews (12) was employed. A sample of the same mixture of substances which was used to obtain the ultracentrifuge pattern depicted in Fig. 4 was subjected to gel filtration on Sephadex G-200. The result (Fig. 6) shows that the sequence of elution of these substances closely reflects their sedimentation behavior, C'3 convertase emerging earlier from the column than C'4i and C'2. Using three reference proteins with known diffusion coefficients, the diffusion coefficients of C'3 convertase, C'4i and C'2 were estimated and found to be 3.3, 3.5, and 4.3×10^{-7} cm^2 sec, respectively (Fig. 7). The values for C'4i and C'2 represent the mean of two determinations. The second gel filtration experiment with C'2 was carried out using partially purified C'2 and isotonic veronal buffer, pH 7.4, containing 0.005 M Na_3EDTA. The difference of the two determinations was less than 3%.

Utilizing the above recorded values for s and D, the molecular weights were calculated and, with the aid of a nomogram for molecular parameters (13), the corresponding frictional ratios were estimated. The partial specific volumes

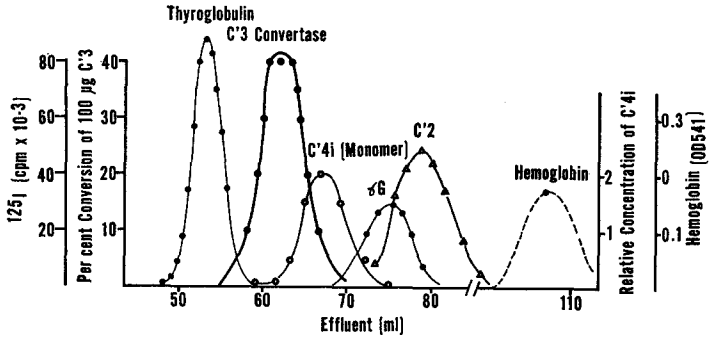

FIG. 6. Elution diagram of C'3 convertase and its precursors, C'2 and C'4i, obtained after gel filtration on a Sephadex G-200 column (2 × 80 cm). The column was equilibrated with phosphate buffer, pH 6.0, ionic strength 0.15. The sample volume was 1.0 ml and also contained three reference substances.

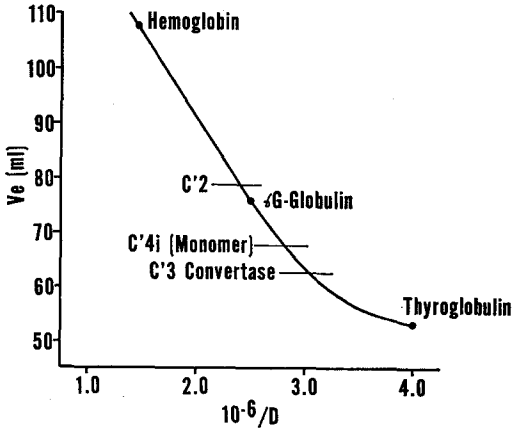

FIG. 7. Estimation of the diffusion coefficients of C'3 convertase and of its precursors, C'2 and C'4i. Elution volumes for gel filtration on a Sephadex G-200 column (see Fig. 6) are plotted against the reciprocal of the diffusion coefficients according to Andrews (12). The diffusion coefficients of three reference substances are: thyroglobulin, 2.5×10^{-7} cm^2/sec; γG-globulin, 3.8×10^{-7} cm^2/sec; and hemoglobin, 6.8×10^{-7} cm^2/sec.

were assumed to be 0.73; this being the value for C'3 which was ascertained on the basis of the amino acid composition of this protein.[2] The results are summarized in Table II. The molecular weight of C'3 convertase, although

[2] H. J. Müller-Eberhard. To be published.

considerably larger than that of C'4i and of C'2, appears to be by 39,000 smaller than the sum of the molecular weights of these two precursors.

TABLE II

Molecular Parameters of C'3 Convertase and its Precursors

Component	*s*	D	f/fo	Mol wt
C'3 convertase	11.2	3.3	1.48	305,000
C'4i (monomer)	8.9	3.5	1.50	229,000
C'2	5.5	4.3	1.50	115,000

s, sedimentation coefficient; D, diffusion coefficient; and f/fo, frictional ratio.

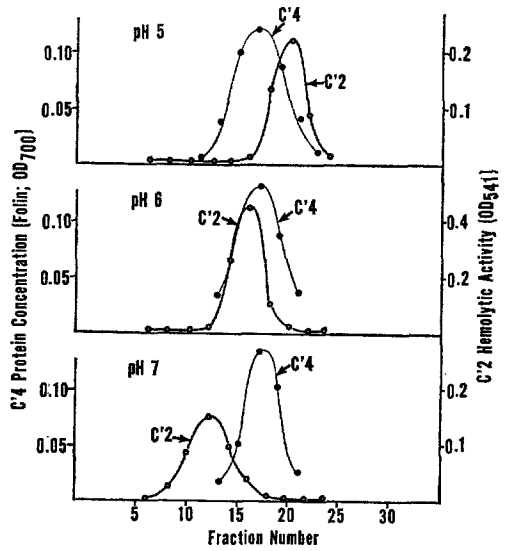

FIG. 8. Demonstration of reversible interaction between purified C'2 and purified C'4 upon ultracentrifugation in a 7–31% sucrose density gradient. Samples of identical composition were applied to the three gradient tubes, each containing 180 μg C'4 and less than 5 μg ^{oxy}C'2 in a total volume of 0.2 ml. Phosphate buffer, ionic strength 0.15, and of indicated pH was present throughout the sucrose gradient. Ultracentrifugation was performed for 20 hr at 35,000 rpm. Direction of sedimentation was toward the left. As marker was used C'3-^{125}I, which is not shown in the diagrams. C'2 was localized by hemolytic assay, C'4 by protein determination.

Reversible Interaction of the Second and Fourth Components of Complement in the Absence of C'1 Esterase.—In control experiments it was found that C'2 is capable of interacting with C'4 and C'4i even in the absence of C'1 esterase. The resulting protein-protein complex differs from C'3 convertase in that it has a greater sedimentation rate, is reversible and lacks C'3-converting activity.

The conclusions were derived from the following observations. When examined by density gradient ultracentrifugation, purified C'2 sediments at a rate of 5.4S–5.7S. In mixtures with purified C'4, in which the concentration of C'4 is large as compared to that of C'2, C'2 sediments appreciably faster, its *s* rate varying with the ionic strength and pH of the buffer. The phenomenon is illustrated in Fig. 8, which shows the ultracentrifugal behavior in mixtures with C'4 at pH 5, 6, and 7. With increasing pH the sedimentation velocity of C'2 becomes greater, while that of the C'4 peak remains unchanged. A similar

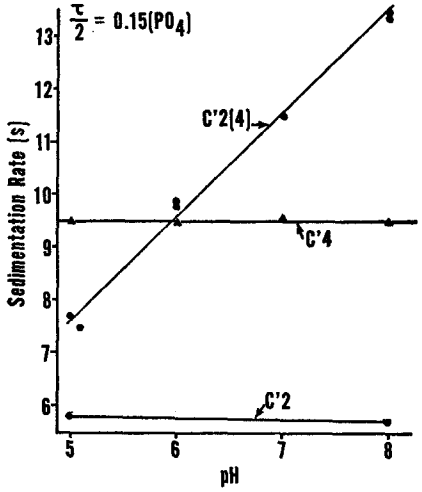

FIG. 9. Plot of sedimentation rates obtained by sucrose density gradient ultracentrifugation against pH, for C'2 alone, C'4 alone, and for C'2 in mixtures with C'4 where the concentration of C'4 was large compared to that of C'2. C'3-^{125}I (9.5S) was used as reference substance for *s*-rate calculations.

pH-dependent variation in *s* rate was not observed when C'2 was analyzed in the absence of C'4.

The results of several experiments show (Fig. 9) that in mixtures of C'2 and C'4, the *s* rate of C'2 varies from 7.7S at pH 5 to 13.5S at pH 8. Under the conditions employed there appears to exist a linear relation between *s* rate and pH. Evidently, at neutral and alkaline pH the interaction between C'2 and C'4 results in the formation of a relatively stable complex which sediments ahead of the zone of free C'4. At pH 5, interaction is minimal causing C'2 to trail behind the C'4 zone, but at a rate greater than that of C'2 sedimenting independently. At pH 6 and 6.5 the interaction causes C'2 to sediment at an intermediate rate and within the C'4 zone.

Similar results were obtained using C'4i-monomer (Fig. 10). The *s* rate of the C'4i,2 complex was smaller than that found for the C'4,2 complex at comparable environmental conditions. This is, most probably, due to the *s* rate

difference between C'4 and C'4i (7). Both the C'4,2- and the C'4i,2-complex are completely devoid of C'3-converting activity.

The effect of ionic strength is recorded in Fig. 11, which shows that the sta-

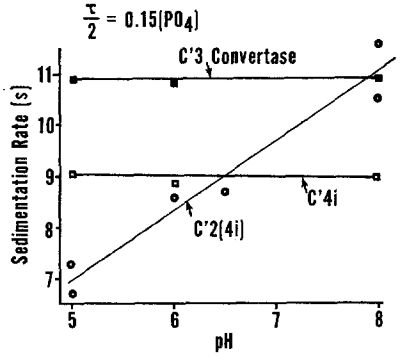

FIG. 10. Plot of sedimentation rates against pH. Analysis by sucrose density gradient ultracentrifugation of C'4i alone and of C'2 in mixtures with C'4i where the concentration of C'4i is large compared to that of C'2. For comparison, data on the ultracentrifugal behavior of C'3 convertase are included.

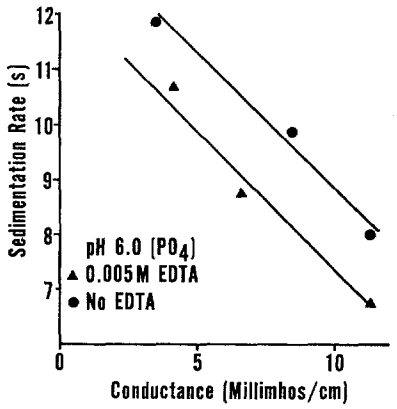

FIG. 11. Sedimentation rate of C'2 in mixtures with C'4 as a function of conductance. The concentration of C'4 was large as compared to that of C'2. Ultracentrifugation was performed in sucrose density gradients.

bility of the complex is inversely proportional to the conductance of the buffer. The presence of 0.005 M Na_3EDTA throughout the sucrose density gradient resulted in weakening of the C'2–C'4 interaction but not in its complete inhibition. It is emphasized that almost all experiments were carried out using phosphate buffers. In a single experiment, isotonic veronal buffer, pH 7.4, was employed. In this case, C'2–C'4 interaction appeared to be completely inhibited by 0.005 M Na_3EDTA and promoted by bivalent cations.

Preliminary results indicate that a similar interaction can also be demonstrated in whole human serum at ionic strength 0.1 or 0.05 in phosphate buffer. After density gradient ultracentrifugation, C'2 was found to be distributed bimodally, the two activity peaks corresponding to *s* rates of approximately 6S and 9S–12S, respectively.

Speed of Formation and Stability of C'3 Convertase Activity.—As mentioned above, the use of ^{oxy}C'2 in the formation of C'3 convertase resulted in a much greater stability of the activity. To compare the chemically modified and the unmodified complex, their formation and decay was studied as a function of

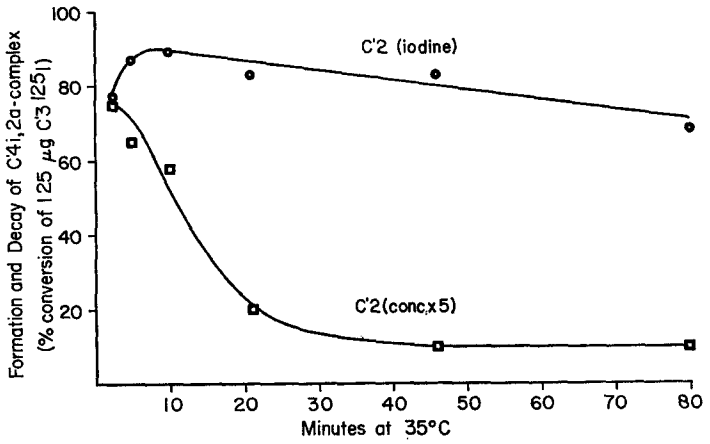

FIG. 12. Formation and decay of C'3 convertase. Activity and stability of C'3 convertase at 35°C are greatly enhanced when it is derived from ^{oxy}C'2 (upper curve). The lower curve was obtained with C'3 convertase derived from unmodified C'2. The latter was used at 5 times the concentration (conc.) of ^{oxy}C'2.

time. Two reaction mixtures were prepared which were identical with respect to the concentrations of C'1 esterase, C'4, and Mg^{++}. One contained a given amount of ^{oxy}C'2 and the other five times this amount of unmodified C'2. Esterase was added last to the reaction mixtures and the time of addition was defined as zero time. Samples were withdrawn after certain intervals and pipetted into a solution of EDTA, thus stopping the process of C'3 convertase formation. Simultaneously, the temperature of the withdrawn sample was lowered to 0°C to prevent decay of the generated activity. At the end of the experiment, C'3 convertase activity was assayed in all samples using C'3-^{125}I. The results are shown in Fig. 12. Three facts are evident. First, formation of convertase at 35°C proceeds rapidly. Second, the complex containing ^{oxy}C'2 is several times as active as the complex prepared with unmodified C'2. Third, the activity of the complex derived from ^{oxy}C'2 is of greater stability, its half-

life at 35°C being approximately 200 min as compared to a half-life of approximately 17 min for convertase derived from unmodified C'2.

Assembly of C'3 Convertase on the Erythrocyte Surface and its Significance in Immune Hemolysis.—Having shown that the (C'4i,2)a complex possesses C'3-converting activity, the question arose as to whether this complex is functionally significant in immune cytolysis. The occurrence of a cell-bound form of the complex appeared probable in view of the previous finding (1) that conversion is a necessary event in the process of binding of C'3 to EAC'1a,4,2a cells. Since direct physicochemical demonstration of cell-bound

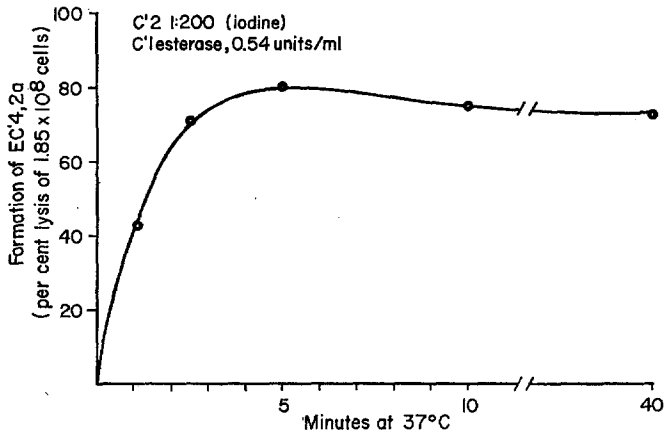

FIG. 13. Formation of erythrocyte-C'4,2a complexes as a function of time. EC'4 cells were treated with ^{oxy}C'2 and C'1 esterase.

C'4,2a is presently not feasible, it was attempted to assemble the complex on the surface of erythrocytes utilizing essentially the same method which was employed for its formation in cell-free solution.

The procedure consists of two steps. First, C'4 was treated with C'1 esterase in the presence of nonsensitized sheep erythrocytes (E). This step, as shown previously (7), leads to formation of erythrocyte-C'4 complexes (EC'4). The cells were washed to remove fluid phase C'4i which otherwise would later compete with bound C'4 for free C'2. During the second step, EC'4 cells were treated with ^{oxy}C'2, Mg^{++} and C'1 esterase. After 5–20 min at 37°C, the cells were washed and analyzed.

Analysis of the resulting product revealed two facts. First, the cells possessed membrane-bound C'3-converting activity and the ability to bind C'3 (1). As they lacked antibody and C'1 because neither of which was supplied, they may be systematically described as EC'4,2a. Second, the cells were readily lysed by EDTA-serum which supplies C'3, 5, 6, 7, 8, and 9, but not C'1, 2, and 4.

The appearance of C'3-converting activity may be considered indicative of successful assembly of the C'4,2a complex on the surface of these cells. Their undergoing lysis in the presence of EDTA-serum demonstrates clearly that the complex thus assembled is hemolytically active. Formation of EC'4,2a from EC'4 and C'2 is shown in Fig. 13 as a function of time at 37°C.

Induction of Hemolysis by Fluid Phase C'3 Convertase.—Ordinarily C'3 convertase is cytolytically effective only when bound to cells. However, in exceptional circumstances cytolysis can be induced by fluid phase C'3 convertase, (C'4i,2)a, although it is incapable of attaching itself to cells. To demonstrate

TABLE III

Induction of Hemolysis by Fluid Phase C'3 Convertase

Treatment	Per Cent Lysis of 2×10^8 Cells	
	PNHE	Norm. Hum. E.
EDTA-C'	0	0
EDTA-C' + (C'4i, 2)a	28	0
EDTA-KSCNC'	0	0
EDTA-KSCNC' + (C'4i, 2)a	40	0
EDTA-buffer + (C'4i, 2)a	0	0

Conditions: C' 1:40, EDTA 0.01 M, volume 1 ml, 40 min at 32°C; (C'4i, 2)a 20 μg (prepared with ^{oxy}C'2).

Abbreviations: PNHE, erythrocytes from patient with paroxysmal nocturnal hemoglobinuria; Norm. Hum. E., normal human erythrocytes; and KSCNC', human serum treated with 1 M KSCN.

the hemolytic activity of fluid phase C'3 convertase erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) were used which are known to be particularly susceptible to complement action (14). As normal erythrocytes, these cells are unaffected by incubation with EDTA-serum. However, when (C'4i,2)a was added which had been prepared with ^{oxy}C'2, hemolysis ensued. The hemolytic effect of (C'4i,2)a could not be explained solely in terms of its action on C'3 because hemolysis was also observed when KSCN-treated serum was employed which contained C'3 in hemolytically inactive form (15). The results which are summarized in Table III indicate that fluid phase C'3 convertase exerts its hemolytic effect by acting on C'3 as well as on later components of the complement sequence.

Effect of C'3 Convertase on C'3 in Cell-Free Solution.—The action of cell-bound C'3 convertase on C'3 has been shown to result in increased electrophoretic mobility, reduced sedimentation velocity, and loss of at least one antigenic determinant. The product, C'3i, was hemolytically inactive. At

neutral or alkaline pH, a low molecular weight split product could not be detected after conversion (1). However, at acid pH a split product becomes dissociated from C'3i, as was first pointed out by Lepow.[3] Fig. 14 shows an immunoelectrophoretic analysis of C'3 after conversion by (C'4i,2)a at pH 6.0. In addition to the main precipitin arc in the α_2-globulin region, a smaller precipitin line is visible which fuses with the main arc. It reveals the presence of a rapidly migrating, antigenically deficient fragment of C'3.

This fragment was investigated further using ^{125}I-labeled C'3. After conver-

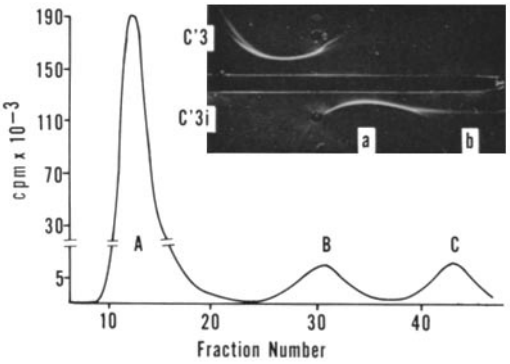

FIG. 14. Demonstration of cleavage of C'3 by C'3 convertase. The curve was obtained by gel filtration on a Biogel P-150 column of C'3-^{125}I after treatment with (C'4i, 2)a. The column was equilibrated with phosphate buffer, pH 5.0, ionic strength 0.15. Component A emerged from the column with the exclusion fraction. The position of cytochrome c (M = 12,000), which was used as reference substance, was between component B and C. Native C'3 gave rise to one fraction only which was excluded by the gel. The inserted immunoelectrophoretic patterns show C'3 before and after conversion. The pattern of C'3i consists of two precipitin lines. That line b merges into line a is interpreted as being due to incomplete dissociation of C'3i under the conditions employed for immunoelectrophoresis.

sion at pH 6.0 C'3 was subjected to gel filtration at pH 5.0. As shown in Fig. 14, three ^{125}I-labeled components emerged from the Biogel P-150 column. The major component comprised more than 90% of the labeled material and appeared in the exclusion fraction. Two minor components, representing together approximately 8% of the labeled protein, were retarded. When native C'3-^{125}I was analyzed at pH 5 using the same Biogel column, the two minor components were not observed, instead all the radioactive label appeared in the exclusion volume.

Using soybean trypsin inhibitor (M = 21,500) and cytochrome c (M = 12,000) as reference substances, it was estimated that the molecular weight of fraction B is between 12,000 and 21,500, and that of fraction C is smaller than

[3] I. H. Lepow. Personal communication.

12,000. It was noticed that the relative proportions of fractions B and C may vary from one experiment to the other. Furthermore, both components tend to aggregate upon storage at 4°C. The chemical nature of the two minor components and their relationship to each other and to the parent C'3 molecule are presently under investigation.

DISCUSSION

As complement represents a system of multiple protein components, its function may be expected to be based upon protein-protein interactions. Interactions between complement components were recently discovered and studied by physicochemical methods. The first component of complement was found to result from the calcium-dependent association of three distinct serum proteins, C'1q, C'1r, and C'1s (16). Purified C'1q was shown to enter into reversible, Ca-independent complexes with native γG- and γM-globulin (17). The fifth, sixth, and seventh components of human complement were recognized as an interacting protein system (18). The present publication reports of yet another example of functionally relevant interactions between complement components. The second and the fourth components were found to interact with each other in two distinct ways; they form reversible complexes in the absence of C'1 esterase and in its presence they give rise to a structurally complex enzyme: C'3 convertase.

The reversible interaction between C'2 and C'4 is readily demonstrated by zone ultracentrifugation. It is dependent upon pH, ionic strength, and other environmental factors such as buffer ions. It is facilitated by a relatively high concentration of C'4. The variability of the sedimentation velocity of C'2 in mixtures with C'4 may be explained in the following manner. The rate constants of the reaction $C'2 + C'4 \rightleftarrows C'4,2$ vary with environmental conditions. Under conditions which favor dissociation, C'2 is enabled to sediment in free form. Conditions that favor association cause C'2 to sediment in the complex. Between these two extremes C'2 may assume a variety of different sedimentation velocities, its actual velocity in a given experiment being a reflection of the prevailing equilibrium position of the interacting C'2–C'4 system.

The reversible reaction between C'2 and C'4 is indicative of a functionally meaningful molecular affinity. These two proteins are precursors of an enzyme which is generated through the action of C'1 esterase and which assumes, for many reasons, a central position in the complement reaction. Since it is acting on C'3, effecting its conversion to C'3i, it was provisionally referred to as C'3 convertase. The complex structure of the enzyme was inferred from two independent observations. First, both C'2 and C'4 are required for the formation of the enzyme. Second, the molecular weight of the active entity is larger than the molecular weight of either C'2 or C'4.

Molecular weight determinations yielded values indicating that the C'3 convertase molecule is by 39,000 smaller than the sum of one C'2 and one C'4 molecule. This finding may be interpreted in two different ways. First, the actual value for C'3 convertase may be 344,000 instead of 305,000 as measured. There is some uncertainty as to the exact diffusion coefficient of the enzyme due to the limitation of the method used for its determination. For larger molecules, gel filtration on Sephadex G-200 yields less reliable results. On the other hand, the actual molecular weight of C'2 may be smaller than the value found in this study. As C'2 enters into a reversible interaction with C'4, the presence of the latter influences both the sedimentation and diffusion coefficient. Although measures were taken to exclude interference by C'4, it is theoretically conceivable that small amounts of C'4 were present during the *s* and D determinations on C'2 and that thus the molecular weight determination resulted in an erroneously high value.

The second interpretation considers fragmentation of at least one of the precursor molecules as part of the mechanism of C'3 convertase formation. Instead of the whole molecule, only a fragment thereof becomes incorporated into the enzyme. While fragmentation of C'4 by C'1 esterase has been excluded (7), fragmentation of C'2 is considered possible and has actually been postulated by Mayer and associates (20). Presently available information does not allow distinction between the two alternative interpretations of the molecular weight data.

The conclusion that the C'3-converting principle is an enzyme is based essentially on two observations. A given amount of (C'4i,2)a is able to convert within a short period of time several thousand times this amount of C'3. Secondly, conversion of C'3 is accompanied by fragmentation of the molecule, the fragments being dissociable at acid pH. While it is evident that the natural substrate of the enzyme is C'3, it is not known as yet which type of chemical bond is attacked.

The concept of the formation of C'3 convertase that emerged on the basis of the present study may be summarized as follows (Fig. 15). C'1 esterase–modified C'4 (C'4i) serves as an acceptor for C'1 esterase–modified C'2. Collision of the two molecules results in the formation of the complex which is endowed with C'3-converting activity. Generation of the enzyme requires Mg ions. While the acceptor activity of C'4i is stable, the ability of C'1 esterase–modified C'2 to combine with C'4i is exceedingly labile. Unless a C'2 molecule collides with an acceptor molecule immediately following its "activation", it will become irreversibly inactivated. "Activation" of C'2 by C'1 esterase may involve cleavage of the molecule into a fragment (A) which is capable of being incorporated into the complex and a fragment (B) which is inert. It is emphasized that so far no direct evidence has been obtained for the existence of the B fragment. By inference, however, its size is estimated to be roughly half that of the A fragment, or one-third of the size of the native C'2 molecule.

C'3 convertase is a labile enzyme, which decays at 32°C with a half-life of approximately 12 min. However, its stability and also its activity are much increased when iodine-treated C'2 is used for the assembly of the enzyme. A similar enhancing effect of iodine has been described for the esterase activity of carboxypeptidase A (19). In the latter case, it is due to iodination, while the effect on C'2 appears to be due to oxidation (5). How oxidation of C'2 can affect the stability of the complex is presently not understood.

Pertinent in this connection are the observations made by Mayer and his associates who studied the behavior of guinea pig C'2 in immune hemolysis (20, 21). According to these authors, C'2 is reversibly adsorbed to EAC'4 cells; adsorption being favored by increase of pH and temperature, and by decrease

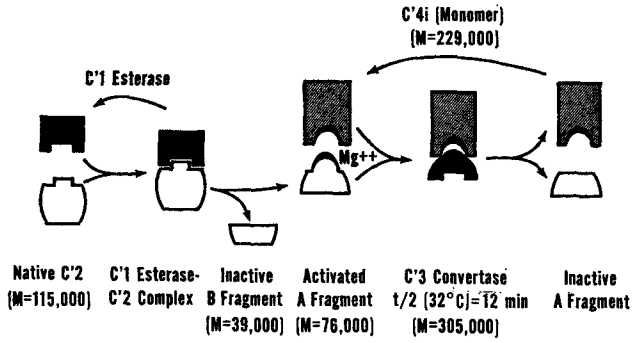

FIG. 15. Postulated mechanism of formation of C'3 convertase.

of ionic strength. These findings are in agreement with, and are fully explained by, the above demonstration of the ability of C'2 and C'4 to interact with each other in free solution and to form reversible complexes. Mayer and associates also found that upon decay of EAC'1a,4,2a to EAC'1a,4, hemolytically inactive C'2 is released which can be detected by an immunochemical method. The molecular weight of the released C'2 was estimated to be 81,000, that of native guinea pig C'2 130,000. The size difference led Mayer to postulate cleavage of C'2 upon fixation during immune hemolysis, a view which is compatible with the data presented in this paper. Borsos and Rapp (22), using only the gel filtration method, reported the molecular weight of C'2 to be 150,000, a value which is probably too high and indicative of C'2–C'4 interaction during measurement.

Regarding the functional significance of C'3 convertase, four pertinent facts may be pointed out. (*a*) When antibody and complement attack a cell the enzyme is assembled on the cell surface where it catalyzes binding of C'3 to the cell (1). (*b*) In addition, it also governs the next reaction step, as the fifth, sixth, and seventh components cannot act on the cell in the absence of C'3 convertase (18). (*c*) As demonstrated above, the enzyme, in its free form, is

capable of triggering lysis of cells from the fluid phase. Although it is an inefficient process, definite cell damage can be caused by this mechanism. It is conceivable that this mode of action of complement is operative in certain situations in vivo. In fact, Yachnin (23) has postulated that intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria is triggered by a "fluid phase" reaction. (*d*) Finally, it is now becoming apparent that C'3 convertase cleaves the C'3 molecule yielding a split product which, through weak forces, remains attached to the parent molecule unless the pH is shifted toward acidity. The possibility is raised that the low molecular weight split product has biological activity. Dias da Silva and Lepow (24) have shown that anaphylatoxin has a molecular weight of less than 60,000 and that C'3 is probably the donor of this histamine-releasing substance. The biological activities of various C'3 fragments are presently under investigation in this laboratory and it will be shown in a subsequent publication that certain pieces of the C'3 molecule effect histamine release from mast cells and attraction of leukocytes.[4]

SUMMARY

A functional unit of the human complement system has been delineated. It is composed of two subunits which are derived from the second (C'2) and from the fourth (C'4) component of complement. Purified C'2 and C'4 were found to interact in free solution and to form a reversible protein-protein complex. When acted upon by C'1 esterase in the presence of Mg ions, the reversible complex acquires stability and the ability to act enzymatically on the third component of complement. The trivial name C'3 convertase has been selected to denote the enzyme. Molecular weight determinations suggest that the entire C'4 molecule, but probably only a fragment of the C'2 molecule, are incorporated into C'3 convertase. Prior treatment of C'2 with iodine led to enhanced stability and activity of the enzyme. It was shown that the cell-bound form of C'3 convertase is cytolytically active and that the free enzyme is able to induce lysis from the fluid phase of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. Evidence was presented that action of C'3 convertase on C'3 results in fragmentation of the molecule.

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