C'1 ESTERASE EFFECT ON ACTIVITY AND PHYSICOCHEMICAL PROPERTIES OF THE FOURTH COMPONENT OF COMPLEMENT*,‡

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Elucidation of the molecular events underlying the phenomenon of immune cytolysis requires analysis of a series of intermediate reaction steps, since at least 10 different serum factors are known to participate in the process (1). These serum factors are commonly referred to as complement. The subject of this report is the reaction mechanism of the fourth component (C'4) of human complement.

C'4 of human serum has previously been isolated and identified by immunoelectrophoresis as β_{1E}-globulin (2). Interaction of C'4 with erythrocyte-antibody complexes (EA) is mediated by the first component of complement (C'1) and corresponds to attachment of β_{1E}-globulin to the cell surface (2). C'1 occurs in serum as a macromolecular complex consisting of three factors (3, 4), one of which represents a proesterase (5) which is activated by interaction of C'1 with antigen-antibody aggregates. The esterase has been shown to be essential for the hemolytic function of activated first component (C'1a) (6, 7). C'1 precedes C'4 in the complement reaction sequence and forms with EA an intermediate complex denoted EAC'1a.

Upon withdrawal of Ca^{++} the C'1 complex is dissociated and its components become separable (3, 4). C'1 esterase could thus be isolated from human serum in highly purified form (7). Addition of the enzyme to serum resulted in rapid inactivation of C'4 (5, 7). Thus, while C'1 esterase is instrumental in the fixation of C'4 when the enzyme is present on the surface of the EAC'1a cell complex, C'1 esterase destroys C'4 activity in free solution. This apparent discrepancy has now been resolved by the finding that the process of inactivation of C'4 is, in fact, closely related to the utilization of this component in immune cytolysis.

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Materials and Methods

Purified Human Serum Components.—C′1 esterase was prepared according to a previously published method (7); the final preparation contained 137 units per ml with a specific activity of 450 units per mg protein. Starch gel electrophoresis of a 10-fold concentrated sample revealed the presence of only one protein component which was located in the postalbumin region. Activity determinations on eluates obtained from segments of part of the starch gel showed that the position of C′1 esterase activity coincided with that of the protein zone.

β_{1E}-Globulin was isolated as previously described (2). However, EDTA in a final concentration of 0.005 M was added to the individual sera, which served as starting material, before these were pooled. This was done to prevent the activation of C′1 esterase, and thus the subsequent inactivation of β_{1E}. The preparation used in this study had a protein content of 4.1 mg per ml and yielded a single homogeneous protein zone upon starch gel electrophoresis. A single precipitin line was observed on immunoelectrophoresis employing antisera to whole human serum and to purified β_{1E}-globulin. The C′4 hemolytic activity of this preparation was similar to that of previous batches (2) (60 per cent lysis of 2.5×10^8 sensitized sheep erythrocytes in the presence of 0.15 μg of β_{1E}, 0.04 ml of hydrazine-treated serum and 5 μg of β_{1C}-globulin in a total volume of 1 ml during incubation at 37°C for 60 minutes).

β_{1C}-Globulin was obtained by a modification of the previous method (8), which will be described in detail in another communication (9). In short, the euglobulin fraction of 500 ml of human serum was first separated by chromatography on a TEAE cellulose column employing a pH and ionic strength gradient (8). Fractions containing β_{1C}-globulin were pooled, concentrated, and the material applied to a hydroxylapatite column equilibrated with 0.1 M phosphate buffer, pH 7.9. Other proteins present were eliminated by elution with starting buffer containing sufficient sodium chloride to raise the conductivity to 13,000 micromhos. Thereafter, β_{1C}-globulin was eluted at a conductivity of 14,500 micromhos. This material was homogeneous, as judged by starch gel electrophoresis and by gel diffusion analysis with an anti-whole human serum.

C′1 esterase inhibitor of human serum was prepared according to Pensky *et al.* (10). The preparation used in these experiments contained 200 units per ml with a specific activity of 25 units per mg protein. 7S γ-globulin was prepared from Cohn fraction II (Lederle Laboratories, Pearl River, New York). This material was first passed through a DEAE cellulose column equilibrated with 0.01 M phosphate buffer, pH 8. The effluent was then subjected to ultracentrifugation for 1 hour at 40,000 RPM in a Spinco SW39 rotor. The concentration of the protein solution was 200 μg per ml. Only the top 2 ml were withdrawn after ultracentrifugation and utilized as "7S" γ-globulin.

Labeling of β_{1E}-Globulin with Radioactive Iodine.—β_{1E}-I^{131} was prepared for quantitative uptake studies of β_{1E}-globulin by EAC′1a cells. Labeling was carried out with carrier free I^{131} using chloramine T and sodium meta bisulfite (11). 500 μc of I^{131} were added to 2 mg of β_{1E} which were contained in 4 ml of ice cold veronal buffer, pH 7.6. With constant stirring 100 μg of chloramine T, from a 200 μg per ml solution in distilled water, were then mixed with the iodine protein solution. Five minutes later 100 μg of sodium meta bisulfite were added from a 200 μg per ml solution and thereafter the protein was dialyzed for 24 hours against 2×10 liters of veronal buffer to remove unbound I^{131}. Using this procedure, uptake of I^{131} varied between 60 and 80 per cent and the specific radioactivity between 3×10^4 and 5×10^4 CPM per μg β_{1E}. C′4 hemolytic activity was at times completely unaffected by the labeling procedure; at other times, however, a reduction in activity was noticed. The reason for the variability of the effect of labeling upon C′4 activity is unknown. The preparation of β_{1E}-I^{131} used in this study had a specific radioactivity of 3×10^4 CPM per μg protein and its C′4 activity had fallen to 30 per cent of the original value.

7S γ-globulin was labeled with I^{131} using the same procedure.

Inactivation of C'1 Esterase.—C'1 esterase was specifically inactivated by incubation with either 10^{-3} M diisopropyl fluorophosphate or an excess of C'1 esterase serum inhibitor. One hour at 37°C was allowed for completion of the reaction.

Inactivation of β_{1E}-Globulin.—Inactivation was achieved by treatment of β_{1E} with C'1 esterase or with dilute hydrazine. 0.1 ml portions containing 400 μg of β_{1E} were treated for 20 minutes at 37°C with C'1 esterase ranging in concentration between 5 and 0.05 units per ml. Hydrazine was used in a final concentration of 0.025 M and inactivation was allowed to proceed for 60 minutes at 37°C.

Antisera to β_{1E}-Globulin.—Antisera were produced in rabbits by injecting each animal twice subcutaneously with 1 mg of purified protein in Freund's adjuvant. A 3-week interval was allowed between the two injections and the animals were bled 2 weeks after the second injection.

Anti-human complement serum containing antibody to β_{1E}- and β_{1C}-globulin was generously furnished by Dr. Karel Pondman, Amsterdam (12).

Preparation of the Intermediate Complexes EAC'1a and EAC'1a,4.—EAC'1a was obtained by incubating washed sheep erythrocytes with 8 hemolysin units of rabbit anti-sheep erythrocyte antibody. The antiserum was not heat inactivated and EDTA was omitted from the reaction mixture to allow not only the antibody, but also the first component of the rabbit antiserum, to attach itself to the cells (13).

The intermediate complex EAC'1a,4 was made by treating 0.4 ml portions containing 2×10^8 EAC'1a cells at 37°C for 30 minutes with varying amounts of β_{1E}-globulin. The total volume was 0.6 ml. Thereafter, the cells were separated from the fluid phase by centrifugation and washed twice in 3 ml veronal buffer.

Determination of C'4 Hemolytic Activity.—To assay C'4 activity in eluates from starch gels and in fractions collected after density gradient ultracentrifugation, appropriate aliquots were incubated with 0.4 ml portions containing 2×10^8 EAC'1a cells. After 30 minutes at 37°C the cells were washed and then set up for hemolysis in a 1 ml volume containing hydrazine-treated human serum in a final dilution of 1:25, and 5 μg of β_{1C}-globulin. After 60 minutes incubation at 37°C, 2 ml of ice cold saline were added to each sample. This was followed by centrifugation and spectrophotometric analysis of the cell-free fluid for free hemoglobin at 541 mμ.

For localization of C'4 activity in a set of fractions after various separation procedures, lysis was not allowed to proceed for 60 minutes, but was interrupted when the sample containing the activity peak approached 60 to 80 per cent hemolysis.

Quantitation of EC'4 Formation.—0.4 ml containing 2×10^8 washed, unsensitized sheep erythrocytes, 0.1 ml β_{1E}, and 0.1 ml C'1 esterase were mixed in this order. In one experiment the amount of β_{1E} was varied between 10 and 100 μg and in another experiment C'1 esterase was varied between 0.00004 and 0.1 unit per ml. Two prior adsorptions of β_{1E} (390 μg per ml) and C'1 esterase (300 μg per ml) with 0.1 volumes of packed unsensitized sheep erythrocytes were without effect on EC'4 formation. The reaction mixtures were incubated for 30 minutes at 37°C, whereupon the cells were washed 3 times in 3 ml veronal buffer. The washed cells were suspended in 0.75 ml veronal buffer and set up for hemolysis with 0.2 ml hydrazine-treated human serum diluted 1:5 in 1/1000 of rabbit hemolysin. 5 μg of β_{1C}-globulin in a volume of 0.05 ml were also added. After 60 minutes at 37°C the samples were analyzed for free hemoglobin.

Controls consisted of samples containing only unsensitized sheep erythrocytes and of samples containing unsensitized erythrocytes and either β_{1E} or C'1 esterase. The low degree of lysis which developed was measured and the values used to correct the optical density readings of the above described set of reaction mixtures (unsensitized cells, β_{1E}, C'1 esterase). The optical density values plotted in Figs. 6 and 7 have been corrected in this fashion.

Immuno- and Starch Gel Electrophoresis.—Immunoelectrophoretic analysis was carried out using the micromethod described by Scheidegger (14). Electrophoresis was performed for 2 hours employing a potential gradient of 5.5 v per cm. Starch gel electrophoresis was performed as described by Smithies (15) using the discontinuous buffer system introduced by Poulik (16)

The protein was applied by means of a filter paper carrier. Electrophoresis was carried out at 4°C for 3 hours in an electric field of 10 v per cm. To localize C′4 activity in the gel, small segments were cut out of one of the two slices usually obtained from a gel. After addition of 0.2 ml veronal buffer, pH 7.6, each segment was mechanically disintegrated and the supernatants, after centrifugation, were used for activity determinations. Freezing and thawing of the segments as a method to elute protein was omitted since this treatment tends to inactivate β_{1E}.

Density Gradient Ultracentrifugation.—This was carried out as described by Kunkel (17). The sucrose gradient was prepared in phosphate buffer, pH 7, T/2 = 0.1; 0.1 ml of test material was applied; ultracentrifugation was performed for 18 to 21 hours at 35,000 RPM at 4°C in a Spinco L-2 machine. Two or three drop fractions were collected from the bottom of the centrifuge tubes.

Conventions.—The term C′1 esterase refers to the enzymatically active subunit of the C′1 macromolecular complex (3, 4, 7). The intact C′1 complex containing activated C′1 esterase is symbolized as C′1a. The protein of human serum representing the fourth component of complement (C′4) is called β_{1E}-globulin; the protein representing the third component (C′3) is designated β_{1C}-globulin. (Serum factors acting later than β_{1C} in the complement reaction are being referred to as C′5, C′6, and so forth.) (1, 18).

The complex between sheep erythrocytes (E) and rabbit antibody (A) is symbolized as EA. The symbol EAC′1a denotes a complex consisting of EA and C′1a. Similarly, EAC′1a,4 denotes a complex consisting of EAC′1a and C′4, and so forth.

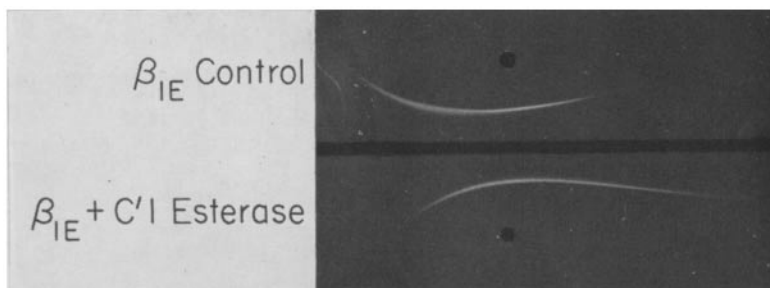

FIG. 1. Immunoelectrophoretic analysis of the effect of C′1 esterase on β_{1E}. 400 μg β_{1E} were incubated for 20 minutes at 37°C with C′1 esterase in a concentration of 0.5 unit per ml. Developed with anti-β_{1E}; anode was at the right.

RESULTS

Changes in Electrophoretic and Ultracentrifugal Properties of β_{1E}-Globulin Caused by C′1 Esterase.—The first question pursued was whether C′1 esterase exerts an effect on the molecular properties of β_{1E}-globulin. Immunoelectrophoresis disclosed that enzyme treatment effected a slight but definite change in electrophoretic mobility (Fig. 1). The treated β_{1E}-globulin exhibited an increased net negative charge and gave rise to an elongated precipitin line which was suggestive of molecular heterogeneity. Two antisera were employed, one prepared to purified β_{1E}-globulin (2), the other to complement-treated immune aggregates (12). In both instances only one precipitin line developed, indicating that an antigenically distinct, precipitable split product had not been produced by treatment with C′1 esterase.

Starch gel electrophoresis revealed a single protein zone for native β_{1E}, but a complex pattern after enzyme treatment (Fig. 2). Two samples which had been incubated with 5 and 0.5 units of C′1 esterase per ml, respectively, gave rise to at least two components, one migrating faster than native β_{1E} and the other more slowly. The component corresponding to native β_{1E} was completely

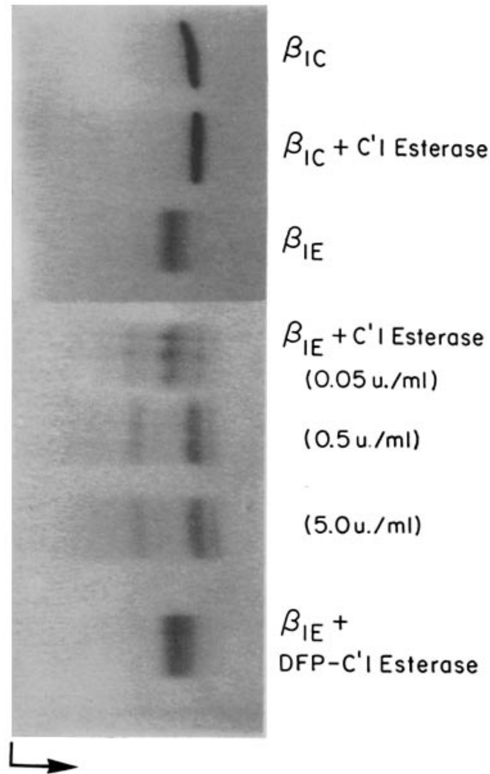

FIG. 2. Starch gel electrophoresis patterns of native and of C′1 esterase-treated β_{1E}, and demonstration of specificity of esterase effect. Each applied sample contained 100 μg of β_{1C}- or β_{1E}-globulin. Anode was at the right.

absent from these two samples, but present in diminished concentration in a third sample which had been incubated with only 0.05 unit of esterase per ml.

The specificity of the esterase effect could be demonstrated in two ways (Fig. 2). First, C′1 esterase inhibited with diisopropyl fluorophosphate (DFP) failed to alter the starch gel pattern of β_{1E}. The same result, not shown in Fig. 2, was obtained with purified serum inhibitor of C′1 esterase (10). Second, the enzyme did not affect the electrophoretic behavior of β_{1C}-globulin, the third component of human complement, an exceedingly labile protein which shares with β_{1E}-globulin the property of being inactivated by dilute hydrazine. The electro-

phoretic mobility of β_{1C}-globulin is known to be changed specifically through the action of the activated second component of complement (19, 20).

Ultracentrifugation in a sucrose density gradient disclosed a reduction in sedimentation rate from 10S to approximately 9.4S for the major portion of enzyme-treated β_{1E} (Fig. 3). A low molecular weight split product was not observed. Instead, material sedimenting considerably more rapidly than the original protein was revealed by the ultracentrifugal analysis, and this is considered to represent complexes consisting of two or more β_{1E} molecules. How these complexes arise is not known, but their possible significance with regard to β_{1E} function will be discussed below. Relating the ultracentrifugal pattern to

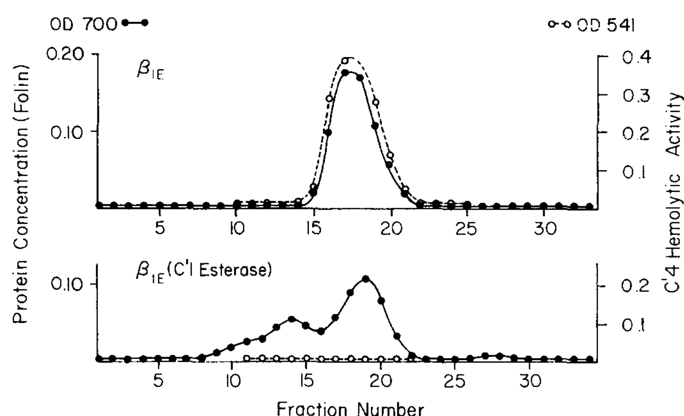

FIG. 3. Analysis of native and C'1 esterase-treated β_{1E} by ultracentrifugation in a 10 to 40 per cent sucrose density gradient after 21 hours at 35,000 RPM. Both patterns were obtained on 410 μg of β_{1E}; esterase treatment was carried out with 0.5 unit per ml at 37°C for 20 minutes. The amount of C'1 esterase added was too small to contribute to the protein curve of the lower pattern. Esterase activity was found in fractions 20 to 28. Direction of sedimentation is toward the left.

that obtained by starch gel electrophoresis, it appears probable that the slowly sedimenting material corresponds to the electrophoretically fast component and the rapidly sedimenting complexes to the electrophoretically slow component.

These results demonstrate the capacity of C'1 esterase to interact with β_{1E}-globulin and to cause distinct changes in its physicochemical properties. They further show that the effect is only produced by the active enzyme and that all molecules of a given preparation of β_{1E}-globulin are susceptible to it.

Correlation Between the C'1 Esterase-Induced Alteration of the β_{1E}-Molecule and Loss of C'4 Activity.—Treatment of β_{1E}-globulin with an appropriate amount of C'1 esterase resulted in complete loss of C'4 hemolytic activity. One microgram of enzyme was found sufficient to inactivate approximately 5000 micrograms

of β_{1E}-globulin in 20 minutes at 37°C. The question arose whether loss of C′4 activity corresponded to the above described changes of the β_{1E} molecule. As shown in Fig. 4, C′4 activity could readily be eluted from the area of the starch gel which was occupied by native β_{1E}. After the complete conversion of this component by 0.5 unit of esterase per ml, C′4 activity could no longer be eluted

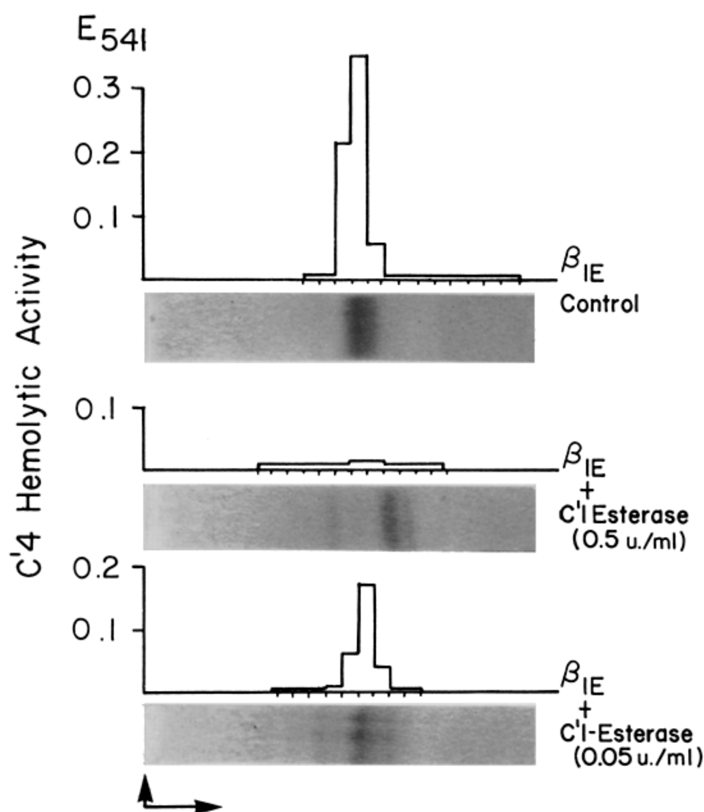

FIG. 4. Demonstration of loss of C′4 activity with conversion of native β_{1E}-globulin by C′1 esterase. C′4 activity was determined after starch gel electrophoresis of native and C′1 esterase-treated β_{1E}-globulin. Each applied sample contained 100 μg of β_{1E}-globulin.

from the now empty area corresponding to the position of native β_{1E}; nor could it be found in segments of the starch gel which contained the electrophoretically faster and slower products of the protein. However, after treatment with 0.05 unit of esterase per ml, which led to only partial inactivation of β_{1E}-globulin, C′4 activity was again recovered. It was found in segments containing the intermediate component of the respective starch gel pattern. The position of the intermediate component corresponded to that of native β_{1E}-

globulin. The possible interference of C′1 esterase in any of the C′4 activity assays can be excluded, since the enzyme has a much greater electrophoretic mobility in starch gel than β_{IE}-globulin and its products.

These observations demonstrate that the β_{IE} molecule becomes hemolytically inactive through conversion by C′1 esterase into its reaction products.

Effect of C′1 Esterase on the Ability of β_{IE}-Globulin to Combine with EAC′1a Cells.—The β_{IE}-molecule must be conceived as possessing at least two functional sites, one enabling the molecule to combine with antibody or with cell membrane receptors, and the other serving as a receptor for the second component of

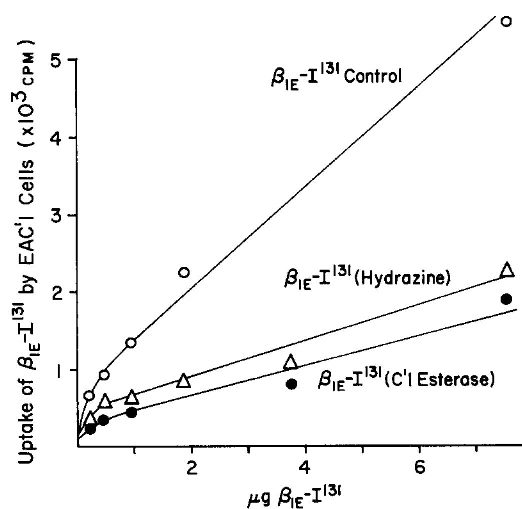

FIG. 5. Effect of C′1 esterase (2×10^{-4} unit per µg β_{IE}) on the ability of β_{IE}-I^{131} to combine with EAC′1a cells. For comparison, the behavior of hydrazine inactivated β_{IE} is also shown. Number of EAC′1a per sample was 2×10^8, total reaction volume 0.7 ml, time at 37°C 30 minutes.

complement. If only the latter site were impaired by C′1 esterase, the resulting molecule, although cytolytically inactive, would probably still be able to combine with EAC′1a cells. If, however, the combining site was affected, uptake by EAC′1a cells should no longer occur. To distinguish between these two possibilities uptake studies were performed employingI^{131}-labeled β_{IE}-globulin. As shown in Fig. 5, uptake of β_{IE}-I^{131} was greatly reduced after C′1 esterase treatment. The degree of residual uptake was similar to that of hydrazine-inactivated β_{IE}-I^{131}, and, in experiments with cells lacking the first component (E or EA), was found to be due to non-specific adsorption of the protein. The data suggest that C′1 esterase acts on the combining site of β_{IE}-globulin. Whether in addition the postulated receptor site for the second component is also affected by C′1 esterase cannot be decided on the basis of this experiment.

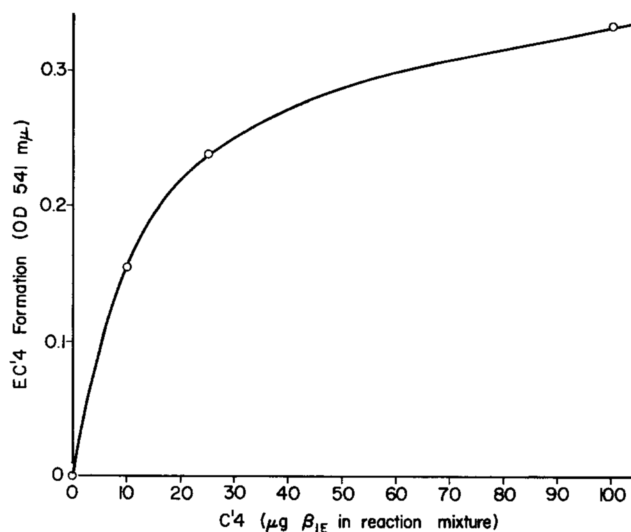

FIG. 6. Formation of EC′4 as a function of β_{1E} concentration in the presence of 0.01 unit of C′1 esterase per ml. Number of E in each sample was 2 × 10^8, reaction volume 0.7 ml, time at 37°C 30 minutes. An optical density reading of 0.3 corresponds to 70 per cent lysis.

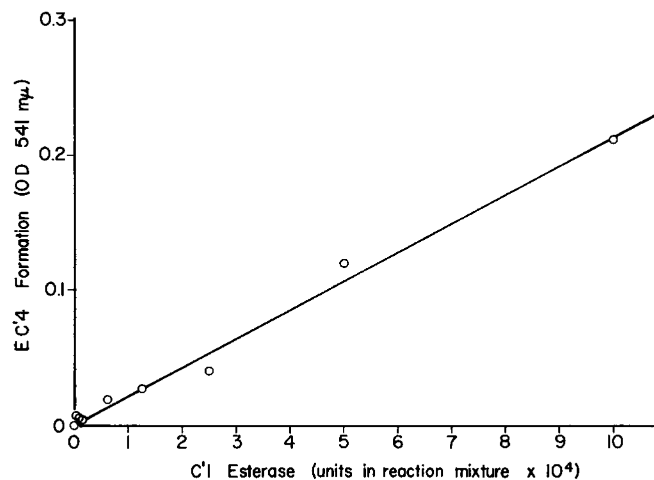

FIG. 7. Formation of EC′4 as a function of C′1 esterase concentration in the presence of 25 μg of β_{1E}-globulin. Number of E in each sample was 2 × 10^8, reaction volume 0.7 ml, time at 37°C 30 minutes. An optical density reading of 0.3 corresponds to 70 per cent lysis.

C′1 Esterase-Induced Formation of Complexes between Non-Sensitized Erythrocytes and Hemolytically Active β_{1E}-Globulin (EC′4).—Inactivation of β_{1E} by C′1 esterase appears to differ from uptake of β_{1E} by EAC′1a cells only in that a suitable receptor for the protein is lacking. If this hypothesis which was first

advanced by Mayer (21) is correct, and if the receptor is not restricted to the antibody molecule, then it should be possible to induce the formation of erythrocyte-β_{IE} complexes (EC'4) in a reaction mixture consisting of non-sensitized red cells, β_{IE}, and C'1 esterase. Formation of EC'4 could, indeed, be demonstrated and is illustrated in Fig. 6 as a function of the amount of β_{IE}-globulin in the reaction mixture, and in Fig. 7 as a function of C'1 esterase concentration. The optical density readings plotted in both figures are corrected as described in Materials and Methods. It is to be emphasized that both preparations were

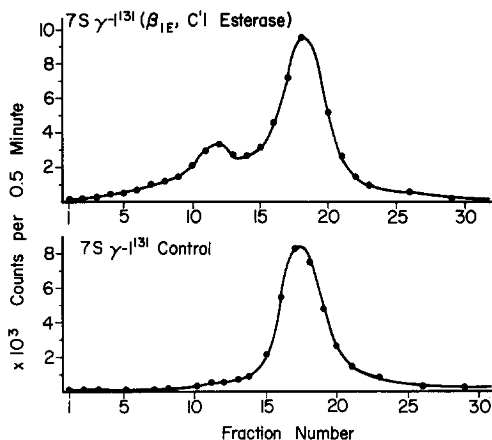

FIG. 8. Demonstration of C'1 esterase-induced β_{IE}-γ-globulin complexes by ultracentrifugation in a 10 to 40 per cent sucrose gradient after 20 hours at 35,000 rpm. Direction of sedimentation is toward the left. Upper pattern was obtained with 20 μg of I^{131}-labeled human 7S γ-globulin after incubation for 10 minutes at 37°C with 800 μg of β_{IE}-globulin and 3.6 units of C'1 esterase per ml in a total reaction volume of 0.3 ml. Lower pattern: I^{131}-labeled human 7S γ-globulin control. Patterns obtained after incubation of 7S γ-globulin with β_{IE} or with C'1 esterase alone were similar to the lower.

adsorbed with non-sensitized sheep erythrocytes to preclude possible participation of small amounts of antibody.

In these experiments, the efficiency of C'4 uptake by non-sensitized cells (E) was less than 1 per cent of that observed with EAC'1a cells, but it increased considerably at higher concentrations of E. Prior treatment of E with C'1 esterase did not result in a larger yield of EC'4, suggesting that the enzyme does not act by preparing the red cell surface for β_{IE}-uptake. Also without effect in the present system was pretreatment of E with neuraminidase, which was previously shown to render non-sensitized human red cells susceptible to autologous complement in the presence of polyethylene glycol (22), and to enhance lysis of sheep erythrocytes by guinea pig complement (23).

The fact that formation of EC'4 complexes is possible indicates that inactivation of β_{IE}-globulin by C'1 esterase is preceded by a state of activa-

tion which enables the β_{1E}-molecule to react with acceptor groups occurring on the surface of erythrocytes.

C'1 Esterase-Induced Formation of Complexes between β_{1E}-Globulin and 7S γ-Globulin.—Although the chemical nature of the β_{1E} acceptor is obscure, it must be inferred from what is known about interaction of complement with antigen-antibody complexes that the distribution of acceptor groups is not restricted to cell membranes. Since it appeared probable that similar groups occur in the γ-globulin molecule and, perhaps, also in other proteins, an experiment was designed to test for the formation of β_{1E}-γ-globulin complexes. To this end, β_{1E}-globulin was treated with C'1 esterase in the presence of I^{131}-labeled human 7S γ-globulin. After incubation for 10 minutes at 37°C, the material was subjected to density gradient ultracentirufgation. As shown in Fig. 8, the distribution of the radioactive label revealed, in addition to a major 7S peak, the presence of a heavier γ-globulin component not present in the original preparation. This heavier material did not arise when γ-globulin was incubated with either C'1 esterase or with β_{1E}-globulin alone. It was therefore concluded that it represents complexes of 7S γ-globulin with β_{1E}-globulin which were induced by the action of C'1 esterase on β_{1E}. The efficiency of complexing was similarly low as that of EC'4 formation.

DISCUSSION

Despite the apparent complexity of the multiple reactions comprising complement action, there is reason to believe that a final understanding will soon emerge in terms of a series of protein-protein and enzyme-substrate interactions. A prerequisite to this end appears to be the opportunity to study the interaction of complement components in simple systems, reduced in composition to 2 or 3 interacting factors. Such studies have become feasible since a number of complement factors have been isolated in recent years from human serum and are now available in highly purified form.

The present paper reports on the action of the purified enzyme moiety of the first component upon the isolated fourth component of human complement. Small amounts of enzyme caused a reduction in s rate of β_{1E}-globulin from 10S to approximately 9.4S, the formation of β_{1E}-β_{1E} complexes, and an increase in net negative charge. While it is not unlikely that the s rate and electrophoretic mobility changes were brought about by the cleavage of a peptide bond, attempts to verify a low molecular weight β_{1E} split product have failed so far. Alternatively, the esterase-induced alterations may be interpreted to arise from conformational changes of the β_{1E}-molecule subsequent to cleavage of a bond without liberation of a split product. The available data do not distinguish between these two possibilities. A third possibility, however, can definitely be ruled out, namely that C'1 esterase splits β_{1E}-globulin into two large fragments. Such a mechanism was suggested by Peetoom and Pondman (24) on the basis

of immunoelectrophoretic observations. These authors analyzed human serum with an antiserum to human complement before and after treatment with C′1 esterase or immune precipitates. They detected distinct changes in the immunoelectrophoretic pattern of the treated sera which were localized to the β-α_2 region. Two precipitin lines designated "β_{1E}-1" and "β_{1E}-2" were thought to be related to two electrophoretically different and antigenically distinct split products of the β_{1E}-molecule. In addition, β_{1E}-1 was found to have antigenic determinants in common with β_{1A}-globulin. The present study, employing purified reactants, shows that C′1 esterase has no such effect on β_{1E}-globulin.

Concomitant with the above described physicochemical changes of β_{1E}-globulin in solution, there occurred complete loss of C′4 activity. The inactivated β_{1E} was no longer taken up by EAC′1a cells and this fact indicated that inactivation by C′1 esterase effects the combining region of the β_{1E}-molecule. What remains uncertain is the fate of the other postulated functional site of the molecule, the receptor for the second component. This site could either be destroyed, unaffected, or generated by the action of the enzyme. Work is presently in progress to clarify this problem.

An unexpected and puzzling finding was the formation of β_{1E} complexes upon treatment of the protein with esterase. Initially, this phenomenon was thought to indicate a tendency of the inactive 9.4Sβ_{1E} product to aggregate. Several observations, however, rendered this interpretation unlikely. It was, for instance, not possible to increase the relative proportion of the heavier material by storage of an inactivated β_{1E} preparation. It appeared more probable that the complexes arose as an immediate consequence of the action of the enzyme on the protein. If the enzyme could cause β_{1E}-globulin to form complexes with itself, then, perhaps, it was also able to initiate the formation of functionally far more significant complexes, namely those between β_{1E} and γ-globulin, or β_{1E} and unsensitized erythrocytes. Both types of complexes were demonstrated above.

The nature of the intermolecular bonds involved has not yet been explored; it is not known, for instance, whether disulfide bonds participate in any of the three types of complexes, nor has it been investigated whether β_{1E} is able to react in a similar fashion with other proteins. It is further unknown whether the bound β_{1E} is still functionally active with respect to interaction with subsequent complement components in all three instances. This could only be determined so far for the erythrocyte-β_{1E} complex which was found to contain the protein in hemolytically active form.

Attachment of β_{1E} to the erythrocyte membrane has also been demonstrated in human autologous systems employing polyethylene glycol instead of antibody (22). Using human cold agglutinin antibody, Harboe (25) obtained similar evidence. Thermal dissociation of the antibody from complement treated EA did not remove β_{1E} from the cells. Utilizing the same system, Boyer (26) found that thermal dissociation of antibody from an EAC′1a, 4 complex was ac-

companied by loss of C′1 activity and production of a residual intermediate complex with properties corresponding to EC′4. It is of further interest that Yachnin (27) has proposed that activation of complement may be effected in the fluid phase by reagents other than antigen-antibody complexes and that such activation may be mediated by an initial reaction between C′1a and C′4 in solution.

While it was shown that the erythrocyte membrane acts as receptor for activated β_{IE}, it remains to be determined to what extent the antibody molecules

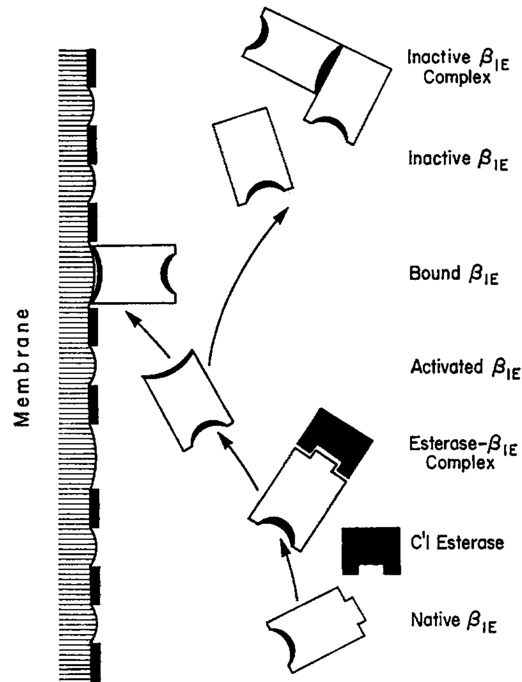

FIG. 9. Schematic illustration of the postulated mechanism of EC′4 formation.

present on EAC′1a cells can serve the same function. Attachment of β_{IE} to antibody is a probable occurrence in immune hemolysis. However, the hemolytic efficiency of a C′4 molecule so placed might differ considerably from that of a molecule attached directly to the cell membrane.

The following tentative concept is derived from the present study (Fig. 9). Action of C′1 esterase on β_{IE}-globulin, a natural substrate, results in activation of this component, enabling it to react with an acceptor occurring on the surface of cells. The term activation refers to induction of chemical reactivity; it does not imply activation of an enzyme. Collision of an activated β_{IE}-molecule with an acceptor group leads to the formation of a β_{IE}-cell complex and the generation of a site on the cell surface which is potentially injurious to membrane

structure and function. If a collision does not occur within a very short period after activation, the reactivity decays and an inactive β_{1E}-molecule ensues. Collision of an activated β_{1E}-molecule with another β_{1E}-molecule may result in the formation of a β_{1E}-β_{1E} complex. In the conventional immune hemolytic system C′1 esterase is not present in free form, but is incorporated in the C′1 macromolecular complex which attaches itself to cell bound antibody molecules. C′1 esterase is thus held in close proximity to the cell surface, a circumstance which greatly increases the probability for a β_{1E}-molecule which has been activated by it to collide and to react with cell membrane groups. That cells in the state EAC′1a are much more efficient in β_{1E}-uptake than E plus C′1 esterase is readily explained by these considerations.

SUMMARY

Highly purified C′1 esterase of human serum is capable of inactivating isolated fourth component of human complement (β_{1E}-globulin). Inactivation is accompanied by changes in electrophoretic and ultracentrifugal properties of β_{1E}-globulin. If non-sensitized sheep erythrocytes are present during the action of C′1 esterase on β_{1E}-globulin, a complex is formed consisting of cells and cytolytically active fourth component (EC′4). Thus, inactivation of β_{1E}-globulin by C′1 esterase appears to be preceded by a state of activation enabling β_{1E}-molecules to combine with cell membrane receptors. Acceptor groups appear to be present also in 7S γ-globulin and in β_{1E}-globulin itself, since C′1 esterase can induce the formation of β_{1E}-β_{1E} and of β_{1E}–7S γ-globulin complexes.

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BIBLIOGRAPHY

1. Müller-Eberhard, H. J., The role of antibody, complement and other humoral factors in host resistance to infections, *in* Bacterial and Mycotic Infections of Man (R. Dubos and J. Hirsch, editors), Philadelphia, Lippincott Publishing Company, 1965, 181.
2. Müller-Eberhard, H. J., and Biro, C. E., Isolation and description of the fourth component of human complement, *J. Exp. Med.*, 1963, **118,** 447.
3. Lepow, I. H., Naff, G. B., Todd, E. W., Pensky, J., and Hinz, C. F., Jr., Chromatographic resolution of the first component of human complement into three activities, *J. Exp. Med.*, 1963, **117,** 983.
4. Naff, G. B., Pensky, J., and Lepow, I. H., The macromolecular nature of the first component of human complement, *J. Exp. Med.*, 1964, **119,** 593.
5. Lepow, I. H., Ratnoff, O. D., Rosen, F. S., and Pillemer, L., Observations on a proesterase associated with partially purified first component of human complement (C′1), *Proc. Soc. Exp. Biol. and Med.*, 1956, **92,** 32.
6. Becker, E. L., Concerning the mechanism of complement action. I. Inhibition of complement activity by diisopropyl fluorophosphate, *J. Immunol.*, 1956, **77,** 462.
7. Haines, A. L., and Lepow, I. H., Studies on human C′1-esterase. I. Purification

and enzymatic properties. II. Function of purified C′1-esterase in the human complement system, *J. Immunol.*, 1964, **92,** 456, 468.

8. Müller-Eberhard, H. J., Nilsson, U., and Aronsson, T., Isolation and characterization of two β_1-glycoproteins of human serum, *J. Exp. Med.*, 1960, **111,** 201.
9. Nilsson, U., and Müller-Eberhard, H. J., Isolation of β_{1F}-globulin from human serum and its characterization as the fifth component of complement, *J. Exp. Med.*, submitted for publication.
10. Pensky, J., Levy, L. R., and Lepow, I. H., Partial purification of a serum inhibitor of C′1 esterase, *J. Biol. Chem.*, 1961, **236,** 1674.
11. Dixon, F. J., and McConahey, P. J., manuscript in preparation.
12. Peetoom, F., and Pondman, K. W., The significance of antigen-antibody complement reactions. III. The identification of C′4 in the immunoelectrophoretic pattern obtained with anti-human complement serum, *Vox Sanguinis*, 1963, **8,** 605.
13. DeLooze, L. L., Ransby, A., and Leon, M., Activity of C′1 in rabbit anti-sheep hemolysin, *Proc. Soc. Exp. Biol. and Med.*, 1962, **109,** 393.
14. Scheidegger, J. J., Une micro-méthode l'immuno-éléctrophorese, *Internat. Arch. Allergy and Immunol.*, 1955, **33,** 11.
15. Smithies, O., Zones electrophoresis in starch gels and its application to studies of serum proteins, *Advances Protein Chem.*, 1959, **14,** 65.
16. Poulik, M. D., Starch gel electrophoresis in a discontinuous system of buffers, *Nature*, 1957, **180,** 1477.
17. Kunkel, H. G., Macroglobulins and high molecular weight antibodies, *Plasma Proteins*, 1960, **1,** 279.
18. Mayer, M. M., Mechanism of hemolysis by complement, *Ciba Found. Symp., Complement*, 1964.
19. Müller-Eberhard, H. J., Calcott, M. A., and Mardiney, M. R., Conversion of β_{1C}-globulin by C′2a, *Fed. Proc.*, 1964, **23,** 506.
20. Pondman, K. W., and Peetoom, F., The significance of the antigen-antibody complement reaction. IV. The transformation of β_{1C}-globulin into β_{1A}-globulin, *Immunochemistry*, 1964, **1,** 65.
21. Mayer, M. M., cited by Rapp, H. J., and Bosros, T., *in* Summary of complement workship, *Science*, 1963, **141,** 738.
22. Dalmasso, A. P., and Müller-Eberhard, H. J., Interaction of autologous complement with red cells in the absence of antibody, *Proc. Soc. Exp. Biol. and Med.*, 1964, **117,** 643.
23. Arquilla, E. R., Hamlin, J., Hamashige, S., and Miller, A., Interference with immune hemolysis by glycoprotein antigens, *J. Exp. Med.*, 1964, **120,** 841.
24. Peetoom, F., Van Der Hart, M., and Pondman, K. W., The significance of antigen-antibody complement reaction. V. The use of cleavage products of human complement in serological studies, *Vox Sanguinis*, 1964, **9,** 85.
25. Harboe, M., Interactions between I^{131} trace-labelled cold agglutinins, complement and red cells, *Brit. J. Haematol.*, 1964, **10,** 339.
26. Boyer, J. T., Stability of the antigen-antibody complex, 10th Congress of the International Society of Hematology, Stockholm, 1964 (abstract).
27. Yachnin, S., Studies on hemolysis of red cells from patients with paroxysmal nocturnal hemoglobinuria, *J. Clin. Inv.*, 1964, **43,** 1240 (abstract).