

# ISOLATION AND ANALYSIS OF THE MECHANISM OF ACTION OF AN INACTIVATOR OF C4b IN NORMAL HUMAN SERUM\*

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This paper documents the existence of an additional complement regulatory principle, C4b inactivator. C4b inactivator, which may well be identical to C3b inactivator, was partially purified from normal human serum and the parameters of its action upon C4b analyzed. C4b inactivator inactivated the hemolytic and immune adherence activity of the activated fourth component of complement, C4b, by direct proteolytic cleavage of the  $\alpha'$ -polypeptide chain of the molecule.

A number of other serum proteins have been identified which act at key sites in the complement sequence to modulate or prevent the progression of the reaction. These regulatory principles include C1 inactivator, a proteolytic enzyme inhibitor that prevents activation of the classical complement pathway by forming a complex with the first complement component and rendering it unable to activate C2 and C4 (1, 2). Biological functions of the activated third component of complement, C3b, are modulated by C3 inactivator or KAF, a serum enzyme that cleaves C3b (3, 4) and which may be identical to C4b inactivator. C3b fragmented by this enzyme is unable to participate in an ongoing complement reaction, to trigger activation of the properdin pathway, or to interact with lymphocytes and other cells possessing receptors for C3b. C3b inactivator has also been postulated to be an inhibitor of Factor D of the alternative pathway (5). The chemotactic and histamine releasing properties of the small cleavage products of C3 and C5, C3a and C5a, respectively, are rapidly lost due to the action of a serum carboxypeptidase B-type enzyme termed anaphylatoxin inactivator (6). Other control proteins have been described (7-11) but these agents have not yet been characterized. The physiological importance of the regulatory proteins of the complement system is indicated by the fact that absence of at least two of them, C1 inactivator and C3b inactivator, leads to uncontrolled activation of the complement system with accompanying disease (1, 5, 12).

## Materials and Methods

*Chemicals and Reagents.* Reagent grade sodium phosphate (J. T. Baker Chemical Co., Phillipsburg, N. J.), sodium barbital (Sigma Chemical Co., St. Louis, Mo.), Tris (Sigma Chemical Co.), and sodium acetate (Mallinckrodt Chemical Works, St. Louis, Mo.) were used to prepare buffers. Phenyl methyl sulfonyl fluoride (PMSF)<sup>1</sup> and diisopropyl fluorophosphate (DFP) were

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<sup>1</sup> *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; PMSF, phenyl methyl sulfonyl fluoride; SDS, sodium dodecyl sulfate.

purchased from Calbiochem, San Diego, Calif. and Boots Pure Drug, Ltd., Nottingham, England, respectively. Agarose was obtained from Marine Colloids Inc., Springfield, N. J. and ion agar from CoLab, Inc., Glenwood, Ill. Sodium dodecyl sulfate (SDS) was obtained from Sigma Chemical Co. and dithiothreitol (DTT) from Bio-Rad Laboratories, Richmond, Calif. The composition of barbital-buffered saline containing  $1.5 \times 10^{-4}$  M calcium,  $5 \times 10^{-4}$  M Mg, and 0.1% gelatin (GVB) has been described (13). Tris-buffered saline was composed of  $5 \times 10^{-2}$  M Tris and  $1 \times 10^{-1}$  M NaCl, pH 7.5.

Soybean, lima bean, and ovomucoid trypsin inhibitors were purchased from Worthington Biochemical Corp., Freehold, N. J., kallikrein-trypsin inhibitor (Trasylol) was obtained through the courtesy of Dr. Schmidt-Kastner, Bayer AG, Elberfeld, Germany. Marker proteins employed for molecular weight determinations included reduced  $\alpha_2$ -macroglobulin (185,000) kindly provided by Dr. Peter C. Harpel, (New York Hospital-Cornell Medical Center, New York) reduced  $\beta$ -galactosidase (135,000) obtained from Worthington Biochemical Corporation, Freehold, N. J., reduced phosphorylase A (94,000) from Sigma Chemical Co., reduced fibrinogen (70,900, 40,400, 49,400) kindly provided by Dr. Michael Mosesson (Downstate Medical Center, State University of New York, Brooklyn, N. Y.), reduced glyceraldehyde-3-phosphate dehydrogenase (36,000) obtained from Worthington Biochemical, and reduced chymotrypsinogen (25,500) obtained from Calbiochem.

Isolated C1 inactivator,  $\alpha_2$ -macroglobulin,  $\alpha_1$ -antitrypsin, and partially purified inter- $\alpha$ -trypsin inhibitor were kindly provided by Dr. Peter C. Harpel. Bovine conglutinin was isolated by the method of Lachmann (14).

*Complement and Complement Reagents.* Fresh human serum was employed for the isolation of C1, C1s, C2, C4, C3, and C4b inactivator. C1s was isolated by a modification (2) of the previously published method (15). C1 (16), C2 (17), C3 (18), and C4 (19) were isolated as described. C2 was exclusively in the oxidized form (20). The cellular intermediates EAC1 (16), EAC14 (17), and EAC1423 (21) were prepared by published methods.

*Preparation of Forssman Antigen-Antibody-Complement Complexes.* Sheep erythrocyte membrane fragments containing Forssman antigen (F) were prepared as described by Rapp and Borsos (22). The particles were sensitized with Forssman antibody, C1, and [ $^{125}$ I]C4 as previously described (19).

*Isolation of C4b Inactivator.* For DE-32 cellulose chromatography, approximately 400 ml of fresh normal human serum was adjusted to pH 7.5 with HCl and dialyzed in the cold against 10 liters of the starting buffer which was composed of  $1 \times 10^{-2}$  M Tris,  $2 \times 10^{-3}$  M EDTA, pH 7.5, conductance 1.2 mmhos/cm (all conductivity measurements were performed at 22°C). After removal from the small precipitate, the supernate was applied to a  $9 \times 60$  cm column of DE-32 cellulose equilibrated with the same buffer. Next, a 2 liter wash was applied followed by a 6 liter NaCl gradient to a limit concentration of 20 mmhos/cm. Fractions containing the peak of C4b inactivator activity were pooled, concentrated by ultrafiltration (Amicon Corp., Lexington, Mass.) to 100 ml and dialyzed against the starting buffer for CM-32 cellulose chromatography. This buffer was composed of  $1 \times 10^{-2}$  M sodium acetate,  $2 \times 10^{-3}$  M EDTA, pH 6.0, conductance 1.1 mmhos/cm. The sample was applied to the column, followed by a 1 liter wash of starting buffer and a 2 liter linear NaCl gradient to a limit concentration of 20 mmhos/cm. The pool of active material was concentrated by ultrafiltration to 50 ml and dialyzed against the starting buffer for hydroxyl apatite chromatography which was  $1 \times 10^{-2}$  M sodium phosphate, pH 7.9, conductance 2.3 mmhos/cm. The sample was applied to a  $2.8 \times 30$  cm column of hydroxyl apatite, followed by a 500 ml wash of starting buffer and a 2 liter gradient composed of 1 liter of starting buffer and 1 liter of sodium/potassium phosphate buffer having a pH of 7.9 and a conductance of 25 mmhos/cm. The limit buffer was prepared by addition of  $6.5 \times 10^{-1}$  M potassium phosphate, pH 7.9 to  $1 \times 10^{-2}$  M sodium phosphate, pH 7.9 to the desired conductance. The active material eluting from the column was concentrated by ultrafiltration to 2 ml and frozen in aliquots at  $-70^\circ\text{C}$ .

*Measurement of Protein Concentration.* Protein was measured by the Lowry procedure (23), with C3 as a reference standard.

*Immunochemical and Electrophoretic Analyses.* Immuno-electrophoresis was performed in 1% agarose in a barbital buffer (pH 8.6) containing  $1 \times 10^{-2}$  M EDTA. Polyacrylamide disc electrophoresis in 6% running gels was performed by the method of Davis (24) in Tris-HCl-glycine buffer, pH 8.7.

*SDS-Polyacrylamide Gel Electrophoresis.* Analyses to determine the molecular weights of C4

and its cleavage fragments were performed in 7% polyacrylamide gels containing 0.1% SDS in phosphate buffer, pH 7.25, according to the method of Weber and Osborn (25), as modified by Harpel and Mosesson (26). 1 vol of protein solution was mixed with 1 vol of phosphate buffer containing 8.5 M urea, 2% SDS, and where indicated,  $1.4 \times 10^{-2}$  M DTT. After an incubation of 45 min at 37°C, an equal vol of 50% glycerol and water saturated with bromophenol blue was added and 25- $\mu$ l samples applied to 6  $\times$  80 or 6  $\times$  130 mm gel columns and electrophoresed for 3 h at 8 mA per gel in a Hoefer gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The electrophoresis buffer was  $1 \times 10^{-1}$  M sodium phosphate, pH 7.25, containing 0.1% SDS. Gels were stained first with Amido Schwartz followed by Coomassie blue. When radiolabeled C4 was employed the gels were sectioned immediately after electrophoresis at 2-mm intervals and the sections analyzed for radioactivity in a Packard auto  $\gamma$ -scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

*Sucrose Gradient Ultracentrifugation.* Samples were sedimented in 7–31% linear sucrose density gradients prepared in barbital-buffered saline, pH 7.3, in an SW-50 rotor at 40,000 rpm for 18 h in a Beckman L-250 ultracentrifuge (Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.). An ionic strength of 0.15 was maintained throughout the gradient. Marker substances were included in separate tubes.

*Radiolabeling of Purified C4.* Isolated C4 was radiolabeled with  $^{125}$ I by the method of McConahey and Dixon (27) as has been described for C4 (19).

*Generation of C3b and C4b.* Isolated C3 was incubated with 2% trypsin (wt/wt) for 5 min at 37°C. Trypsin was neutralized with a twofold excess of soybean trypsin inhibitor and the mixture subjected to electrophoresis in a Pevikon block at pH 8.6. Eluates of the block, which had the ability to induce consumption of C3 on addition to serum were pooled at C3b. Isolated C4b was incubated with a 1:10 molar ratio of isolated C1s for 30 min at 37°C. Verification of C3b and C4b formation was demonstrated by hemolytic measurement which indicated that greater than 99% of the activity of each protein had been lost.

*Measurement of C3 and C4 Hemolytic Activity.* The hemolytic activity of C3 and C4 was measured by effective molecule titration (16, 21).

*Measurement of C3 and C4 Immune Adherence Activity.* Immune adherence activity of bound C4 was assessed by the microtiter method employing dilutions of EAC14 with human type O, Rh erythrocytes as indicator particles (28).

## Results

*Isolation of C4b Inactivator.* The method employed for partial purification of C4b inactivator was adapted from that published by Lachmann for C3b inactivator (3). Initial separation of whole serum into euglobulin and pseudoglobulin fractions was avoided during the isolation procedure since C4b inactivator is present in both fractions. As shown in Fig. 1, fractions from two distinct areas of the chromatogram had the ability to inhibit C4b-dependent immune adherence. Fractions from both areas also inhibited C3b-dependent immune adherence. The first peak of activity emerged from the column just before, and overlapping with C3 proactivator and transferrin in fractions having a conductance of 5.5–7 mmhos/cm. The second area of the column having the ability to inhibit C4b immune adherence correlated with the appearance of C3 from the column. This second inhibitory area was not studied further, as the inhibition was felt to be due to the presence of C3b in the column fractions. Fractions representing the first peak of activity were pooled, concentrated, and subjected to chromatography on CM-cellulose. C4b inactivator activity and also C3b inactivator activity emerged from this column just before and overlapping transferrin in fractions having a conductance of 3 to 6 mmhos/cm (Fig. 1). Complete separation from C3PA, which eluted considerably later, was achieved by using this column. Hydroxyl apatite chromatography removed additional contami-

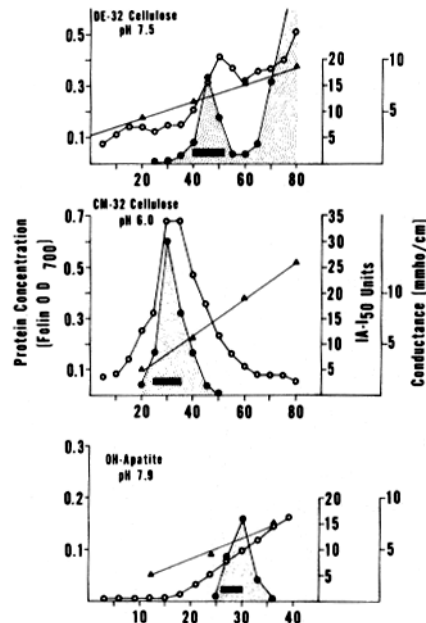


FIG. 1. Chromatographic steps in the isolation of C4b inactivator. The first peak of C4b inactivator activity emerged from DE-32 cellulose (upper panel) at a conductance of 6 mmhos/cm as indicated. The active fractions were pooled, dialyzed, and applied to a CM-32 cellulose column (middle panel). C4b inactivator eluted just before the main protein peak at a conductance of 4 mmhos/cm as indicated. The active fractions were pooled, dialyzed, and applied to an OH-apatite column (lower panel) from which C4b inactivator eluted at a conductance of 6 mmhos/cm.

nants and reduced the contamination of transferrin which eluted before C4b and C3b inactivator activities; these came together at a conductance of 5.5–6.5 mmhos/cm. Although C4b inactivator was not pure at this stage, activity was not detectable after additional chromatographic procedures designed to remove the contaminating Ig and transferrin. Among the separatory procedures attempted were chromatography on Sephadex G-200, QAE-Sephadex, and Biorex-70.

*Physicochemical Characteristics of C4b Inactivator.* Ouchterlony analyses showed the preparations to be unreactive with antisera to C1q, C3, C4, C5, and C3 proactivator, but reactive with antisera to IgG, IgA and transferrin. Polyacrylamide gel electrophoretic analysis of a typical C4b inactivator preparation revealed three bands as shown in Fig. 2. The cathodal of these was due to IgG while the anodal represented transferrin. As indicated in Fig. 2, C4b inactivator activity migrated in parallel unstained gels in the position occupied by the middle band. Although not shown, C3b inactivator activity was also evident in these eluates.

C4b inactivator as well as C3b inactivator migrated as  $\beta_1$ -globulin on electrophoresis in agarose at pH 8.6. Sucrose gradient ultracentrifugal analysis indicated a sedimentation rate of 4.5S for C4b inactivator. C4b and C3b inactivator activities eluted together with transferrin from Sephadex G-200 columns, indicating a diffusion coefficient of approximately  $5.3 \times 10^{-7}$  cm<sup>2</sup>/sec. A mol wt of approximately 88,000 daltons was calculated from the S-rate and

diffusion coefficient. C4b- and C3b-inactivating activities were destroyed by incubation with  $1 \times 10^{-2}$  M DTT at pH 8.0 but not by  $1 \times 10^{-2}$  M DFP or PMSF, 100  $\mu\text{g}/\text{ml}$  of soybean, lima bean, ovomucoid or kallikrein trypsin inhibitors, or by 5  $\mu\text{g}/\text{ml}$  of isolated C1 inactivator,  $\alpha_2$ -macroglobulin,  $\alpha_1$ -antitrypsin, or inter- $\alpha$ -trypsin inhibitor.

*Effect of C4b Inactivator on Cell-Bound C4b.* Varying amounts of C4b inactivator were incubated with  $2 \times 10^8$  EAC14 in a total vol of 0.1 ml for 60 min at 37°C. After washing, active C4b remaining on the cells was assayed hemolytically and by immune adherence. As indicated in Fig. 3, C4b inactivator produced a dose-dependent loss in the hemolytic and immune adherence activity of bound C4b. Parallel studies with EAC1423 prepared with limited amounts of C4 which were immune adherence-negative at the EAC14 stage showed that the C4b inactivator preparations also inactivated the hemolytic and immune adherence activity of bound C3b. Similar studies performed with EAC1 [ $^{125}\text{I}$ ]C4 showed a dose-dependent reduction in cell-bound C4 radioactivity with 19% of the total counts being dissociated by the largest amount of C4b inactivator employed, 87  $\mu\text{g}$ .

The relationship between inactivation of bound C4b activity and dissociation

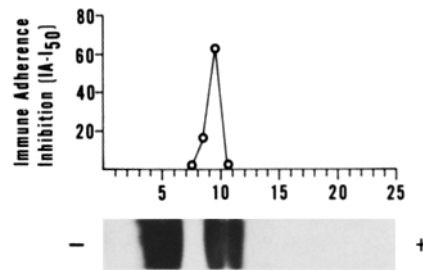


FIG. 2. Mobility of C4b inactivator on electrophoresis in 6% polyacrylamide gels. C4b inactivator was eluted from segments of a parallel unstained gel.

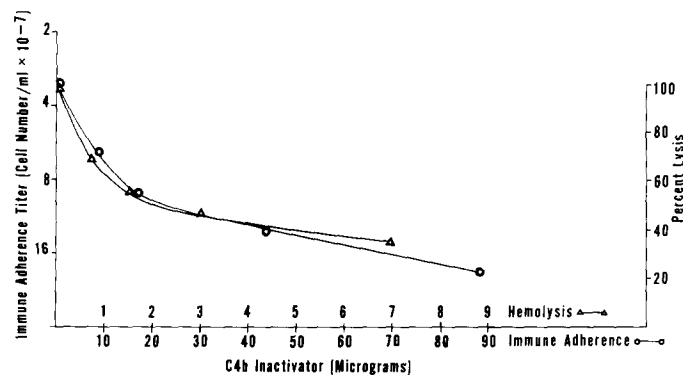


FIG. 3. Dose-dependent inactivation of the hemolytic and immune adherence activity of cell-bound C4b by C4b inactivator. Hemolytic testing ( $\Delta$ - $\Delta$ ) showed 50% inactivation of a limited amount of cell-bound C4b by 1.5  $\mu\text{g}$  of C4b inactivator, while 50% inactivation of the immune adherence activity (O-O) of cells bearing a larger amount of C4b, approximately 5,000 molecules per cell with 10  $\mu\text{g}$  of C4b inactivator.

of radiolabeled C4 from cells was determined by periodic samplings at 37°C of a reaction mixture composed of EAC1 [ $^{125}$ I]C4 and 46  $\mu$ g of C4b inactivator. As indicated in Fig. 4, immune adherence activity was rapidly lost, whereas C4 radioactivity was released from the cells at a considerably slower rate. This difference in kinetics suggests that functional inactivation is not the direct result of dissociation of C4b from the cells. A maximum of 70% of the bound C4 radioactivity could be removed after prolonged incubation (37°C, 24 h) with large amounts of C4b inactivator. In order to determine whether the failure to completely remove bound C4 was due to cleavage of the molecule with retention of a fragment on the cell, [ $^{125}$ I]C4 eluted from EAC1 [ $^{125}$ I]C4 by C4b inactivator was examined by sucrose gradient ultracentrifugation. As shown in Fig. 5, a 5.5S

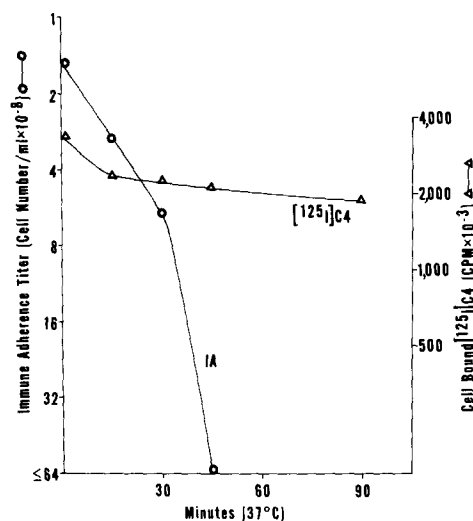


FIG. 4. Kinetic study of the ability of C4b inactivator to dissociate C4 radioactivity from EAC1 [ $^{125}$ I]C4 and to inactivate C4b-dependent immune adherence activity.

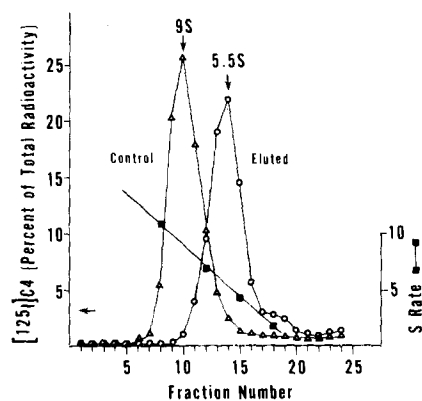


FIG. 5. Sucrose gradient ultracentrifugal analysis of C4b and of [ $^{125}$ I]C4 dissociated from EAC1 [ $^{125}$ I]C4 by C4b inactivator. Marker proteins employed to determine the sedimentation rate were C1q, IgG, human serum albumin, and cytochrome C.

sedimentation rate was observed for C4 eluted by the action of C4b inactivator in contrast to the 9S rate observed for C4b, a finding which documents cleavage of bound C4 by C4b inactivator.

*Effect of C4b Inactivator on C4b in Solution.* C4b was incubated with buffer or with C4b inactivator for prolonged periods of time (up to 24 h) at 37°C. The status of C4b was then examined by immunoelectrophoresis in agarose. A new anodally migrating line reactive with anti-C4 appeared after treatment with C4b inactivator. Residual C4b appeared to spur over this derivative line.

*Effect of C2 on the Susceptibility of EAC14 to C4b Inactivator.* EAC14, bearing approximately 8,000 molecules of C4 per cell, were incubated with various concentrations of oxidized human C2. After washing, batches of EAC142 cells were incubated with C4b inactivator or with buffer for 60 min at 37°C, washed again, and examined for immune adherence activity. EAC14 which had not been incubated with C2 showed a decrease in immune adherence titer from  $1 \times 10^7/\text{ml}$  to  $>3 \times 10^8/\text{ml}$  after treatment with C4b inactivator as shown in Fig. 6. In contrast, the ability of C4 inactivator to attack bound C4b was impaired by the presence of cell-bound C2. Thus, EAC142 prepared with the highest dose of oxidized C2 were almost completely protected from C4b inactivator action. A progressive increase in susceptibility to C4b inactivator was observed as the amount of bound C2 was decreased as indicated in Fig. 6. Interestingly, the presence of C2 on EAC14 did not affect the ability of the C4 to participate in immune adherence, since EAC14 and the various batches of EAC142 had the same immune adherence titer.

*Effect of C4b and C3b on C4b Inactivator.* Since C4b inactivator acted upon C4b in free solution or on the surface of cells, it was considered likely that C4b in solution would inhibit the attack of C4b inactivator on bound C4b by providing

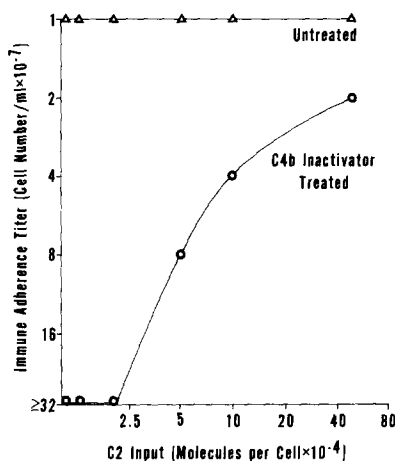


FIG. 6. Effect of C2 on the susceptibility of the EAC14 to inactivation by C4b inactivator. Full inactivation of cell-bound C4b immune adherence activity was obtained on treatment of EAC14 and EAC142 bearing small amounts of cell-bound C2 with C4b inactivator. With increasing amounts of cell bound C2, the cells became progressively more resistant to inactivation by C4b inactivator. Cell bound C2 did not influence the reactivity of cell bound C4b in immune adherence as shown by the identical titer of the various batches of EAC142.

additional substrate. Varying amounts of C4b were added to a mixture of C4b inactivator and EAC14. After 60 min at 37°C the cells were washed, diluted, and examined for immune adherence activity. In the control, which did not contain C4b, C4b inactivator reduced the  $IA_{50}$  titer of the cells from  $1 \times 10^7/ml$  to  $>2 \times 10^9/ml$ . The action of C4b inactivator on EAC14 was inhibited by the presence of C4b as shown by an increase in the  $IA_{50}$  titer to  $1.25 \times 10^8/ml$ ,  $2.5 \times 10^8/ml$ , and  $1 \times 10^9/ml$  when 145, 72, and 36  $\mu g$  of C4b, respectively were included. In the same experiment C3b was also found to inhibit the action of C4b inactivator on EAC14.  $IA_{50}$  titers of  $1 \times 10^7/ml$ ,  $3 \times 10^7/ml$ ,  $2 \times 10^8/ml$ , and  $1 \times 10^9/ml$  were obtained when 96, 48, 24, and 12  $\mu g$  of C3b, respectively, were present during the reaction of C4b inactivator with EAC14.

*Lack of Reactivity of C4b Inactivator-Treated Cell-Bound C4b with Conglutinin.* C3b inactivator has been termed KAF or conglutinin-activating factor because of its ability to render bound C3b reactive with bovine conglutinin (3). The following experiment was performed to determine whether bound C4b was also capable of acting as a conglutinin after treatment with C4b inactivator. EAC14 were incubated with dilutions of C4b inactivator for 60 min at 37°C after which purified bovine conglutinin (300  $\mu g/ml$ ) was added. After 30 min of incubation at 37°C the cells were allowed to settle and the pattern of the sedimented erythrocytes determined. No agglutination (conglutination) of the cells and thus no reactivity of the EAC14 with conglutinin was evident. In parallel experiments with EAC1423, the same amount of conglutinin strongly agglutinated the cells to a 1:800 dilution of the C4b inactivator preparation. Thus, C4b is unable to act as a conglutinin after treatment with C4b inactivator.

*Analysis of the Effect of C4b Inactivator on the Subunit Structure of C4.* In order to determine the nature of the attack on the C4 molecule by C4b inactivator, radiolabeled preparations of C4 and C4b were incubated with C4b inactivator. Urea, SDS, and DTT were added, and the mixtures were subjected to electrophoresis in SDS-polyacrylamide gels. The gels were sectioned and the segments analyzed for radioactivity. The basic three polypeptide chain structure of C4 was not found to be altered by incubation with C4b inactivator. Mol wt for the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains were 96,000, 74,000 and 32,000 daltons, respectively, in good agreement with earlier studies (19). In contrast, the structure of C4b was markedly altered by C4b inactivator. In the experiment depicted in Fig. 7, long SDS-polyacrylamide gels were employed in order to better define the effects. The average mol wt obtained in these studies for the  $\alpha'$ -,  $\beta$ - and  $\gamma$ -chains were 84,000 (range 80,000–87,000), 76,000 (70,000–79,000), and 32,000 (29,000–36,000) daltons, respectively (Fig. 7, upper panel). As shown in the central panel, C4b inactivator markedly diminished the amount of radioactivity associated with the position of the  $\alpha'$ -chain of the molecule. In addition, a new peak having an average mol wt of 49,000 (46,000–52,000) daltons appeared, and the percentage of total radioactivity present in the 30,000–35,000 dalton range occupied by the  $\gamma$ -chain increased. Furthermore, the percentage of total radioactivity present in the 49,000 dalton peak plus the increment in radioactivity of the 30,000–35,000 dalton range approximately equalled the percentage of radioactivity missing from the  $\alpha'$ -peak. From these studies it is evident that



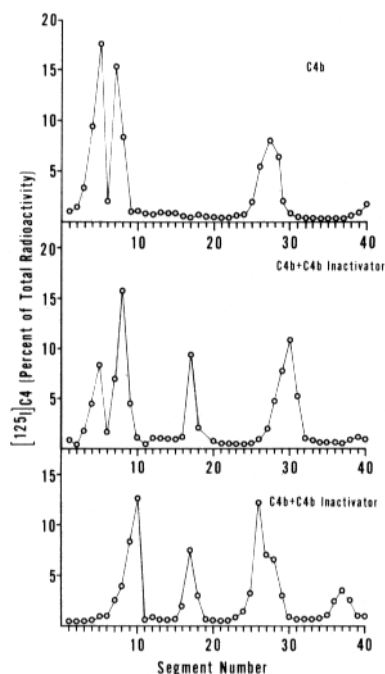


FIG. 7. Effect of C4b inactivator on the structure of C4b. C4b was incubated alone (upper panel), with a purified C4b inactivator preparation (middle panel) or with a crude preparation of C4b inactivator (lower panel) after which the mixtures were analyzed by electrophoresis in SDS acrylamide gels. The purified C4b inactivator preparation produced a decrease in the amount of radioactivity associated with the  $\alpha'$ -chain of C4b (middle panel). The radioactivity missing from the position of the  $\alpha'$ -chain was accounted for by a cleavage product having a mol wt of 49,000 daltons and by an increase in the percentage of the total radioactivity present in the 30,000–35,000 dalton range occupied by the  $\gamma$ -chain. Crude C4b inactivator preparations (lower panel) in addition cleaved the  $\beta$ -chain into a major fragment having a mol wt of 67,000 daltons and a minor fragment or fragments having a mol wt of less than 20,000 daltons.

C4b inactivator cleaves the  $\alpha'$ -polypeptide chain of C4b into fragments having mol wt of 40,000 daltons and approximately 30,000–35,000 daltons. Since no smaller fragments bearing radioactivity were detected, it may be assumed that C4b inactivator cleaves the  $\alpha'$ -chain at a single site producing two fragments having mol wt of 49,000 and 35,000 daltons, respectively. This structural alteration undoubtedly represents the mechanism by which C4b inactivator destroys the functional activity of C4b.

A second cleavage in the  $\beta$ -chain of the C4b molecule (Fig. 7, lower panel) was produced by crude C4b inactivator preparations that had only been subjected to the first step of the isolation procedure. This second cleavage was never observed with the more highly purified C4b inactivator preparations. The second cleavage generated a large fragment having a mol wt of 67,000 daltons and a smaller fragment or fragments which had a mol wt of less than 20,000 daltons. This secondary cleavage reaction was not essential for inactivation of C4b functional activity since preparations of C4b inactivator which produced only cleavage of the  $\alpha'$ -chain rendered bound C4b functionally inactive. In order to determine

which fragment of the  $\alpha'$ -polypeptide chain remained on the cell after treatment with C3b inactivator, an experiment with Forssman antigen particles sensitized with antibody, C1, and [ $^{125}$ I]C4 was performed. FAC1 [ $^{125}$ I]C4 complexes were incubated with a crude preparation of C4b inactivator for 60 min at 37°C. After washing, the adherent C4b fragment was dissociated by SDS and analyzed by SDS acrylamide gels. Most of the radioactivity was present in a peak having a mol wt of 35,000 daltons as indicated in Fig. 8. A minor peak with a mol wt of 45,000 daltons was also observed. This study indicates that the smaller fragment of the  $\alpha'$ -chain remained adherent to complexes after treatment with C4b inactivator and thus may be termed C4d in analogy to the comparable fragment of C3. In the same study, the C4 fragment dissociated from FAC [ $^{125}$ I]C4 by C4b inactivator migrated as a single peak of radioactivity on electrophoresis in SDS acrylamide gels in the absence of DTT. After reduction and analysis of

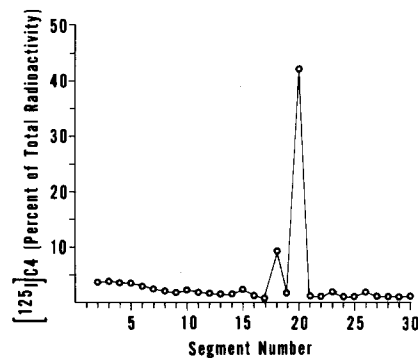


FIG. 8. SDS acrylamide gel electrophoretic analysis of the fragment of C4 remaining bound to FAC1 [ $^{125}$ I]C4 after extensive treatment with C4b inactivator. The adherent fragment dissociated by SDS had a mol wt of 35,000 daltons. In addition, a second minor peak having a mol wt of 45,000 daltons was observed.

this fragment, which may be termed C4c, in SDS acrylamide gels, two broad peaks of radioactivity were observed which had mol wt at their highest points of 64,000 and 29,000 daltons, respectively.

### Discussion

This paper documents the existence of an inactivator of C4b in normal human serum. The active principle was isolated in a partially purified form from serum and found to be a  $\beta_1$ -globulin with an approximate mol wt of 88,000 daltons. C4b inactivator acted only upon the activated fourth component of complement, C4b; native C4 was completely resistant to its action. Incubation of bound C4b with C4b inactivator resulted in a time and dose-dependent inactivation of the hemolytic activity of bound C4b. In addition, the ability of bound C4b to interact with erythrocytes or lymphocytes (29) which possess receptors for C4b, was also lost.

C4b inactivator abrogated the biological activity of C4b by cleaving it into two fragments which may be termed C4c and C4d in analogy to the terminology used

for C3. Cleavage of the molecule could be demonstrated directly on immunoelectrophoretic analysis of inactivator-treated C4b. The fragments separated after treatment of cell-bound C4b with C4b inactivator followed by prolonged incubation, since C4c was dissociated from the cells under these conditions while C4d remained adherent. C4c was found to sediment as a 5.5S protein in sucrose gradients in contrast to the 9S rate observed for C4b. C4d remaining adherent to the cells was detected by residual radioactivity after extensive treatment with C4b inactivator and, although not described here, by agglutination of the treated cells with antisera which detected the fragment.

SDS-polyacrylamide gel analyses of inactivator-treated C4b revealed a single cleavage site located in the  $\alpha'$ -polypeptide chain of the molecule. Cleavage at this site represents the mechanism of inactivation of the functional activity of C4b since the more purified preparations of C4b inactivator produced only this cleavage. The two fragments of the  $\alpha'$ -chain, termed  $\alpha_2$  and  $\alpha_3$ , had mol wt of 35,000 and 49,000 daltons, respectively (Table I). The  $\alpha_2$ -fragment was identified as C4d by elution from FAC1 [ $^{125}\text{I}$ ]C4 extensively treated with C4b inactivator.

TABLE I  
Fragments of C4 and of C4 Polypeptide Chains

C4a = $\alpha_1$		
C4b = $\alpha' (\alpha_2 + \alpha_3) + \beta + \gamma$		
C4c = $\alpha_3 + \beta + \gamma$		
C4c' = $\alpha_3 + \beta_2 + \gamma$		
C4d = $\alpha_2$		
$\alpha$ -Chain (90,000)	$\beta$ -Chain (76,000)	$\gamma$ -Chain (32,000)
$\alpha_1$ (6,000)	$\beta_1$ (9,000)	
$\alpha_2$ (35,000)	$\beta_2$ (67,000)	
$\alpha_3$ (49,000)		

C4c is thus composed of the remaining portion of the  $\alpha$ -chain,  $\alpha_3$ , plus the  $\beta$ - and  $\gamma$ -chains. Since C4b inactivator treatment alone dissociated C4b, the  $\alpha_2$ -fragment is not disulfide-linked to the remainder of the molecule. The three polypeptide chains of C4c, however, are linked by disulfide bridges since C4c sediments in sucrose gradients and migrates in polyacrylamide gels as a single peak of radioactivity in the absence of reducing agents. These concepts are illustrated schematically in the model for C4 structure shown in Fig. 9.

Crude preparations of C4b inactivator produced, in addition, a second cleavage in the  $\beta$ -chain of C4b. A reduction in the mol wt of the  $\beta$ -chain from 76,000 daltons to 67,000 daltons and the concomitant appearance of a small fragment, or fragments, migrating close to the buffer front were observed on treatment of C4b with crude C4b inactivator preparations. Although it is not clear that this was a single cleavage, it is depicted as such in the model (Fig. 9). This cleavage was not essential for inactivation as considered above. It is not known whether this secondary cleavage was also produced by C4b inactivator, since the crude preparations were considerably more active with regard to hemolytic and immune adherence inactivation than the more highly purified preparations. The crude



### Summary

A complement regulatory principle, C4b inactivator, was isolated in a partially purified form from normal human serum. The C4b inactivator, a  $\beta_1$ -globulin with an approximate mol wt of 88,000 daltons, and which may be identical to C3b inactivator, cleaved C4b in free solution or on the surface of cells and rendered it unable to participate in hemolytic reactions or to interact with cells having receptors for C4b. C4b inactivator functioned by cleaving the  $\alpha'$ -polypeptide chain of C4b at a single site which was sufficient to dissociate the molecule into two fragments, C4c and C4d, and to inactivate its biological function. Certain structural correlates of C4 functions deriving from these studies are discussed and a model for C4 structure based on these findings is presented.

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