The C3b inactivator (C3bINA)\(^1\) has been reported to cleave and inactivate cell-bound C3b (1, 2), C3b in solution (3), and cell-bound and free C4b (4, 5). In this capacity it constitutes an important regulatory protein of the classical (6) and alternative complement pathways (7, 8). Its physiological role in complement homeostasis has become apparent through the observation of two cases of homozygous C3bINA deficiency (9, 10), which are associated with C3 hypercatabolism and increased susceptibility to severe bacterial infections (11). Recently, the serum protein \(\beta 1H\) (12) has been identified as an accelerating cofactor (13) of C3bINA and has been shown to have, in addition, independent regulatory functions in the alternative pathway (8, 14).

Although the enzyme was described as C3bINA in 1966 (1) and as conglutinogen activating factor in 1968 (2), the enzyme has not been obtained heretofore as a homogeneous protein. As a consequence, the precise mechanism of action on C3b and C4b could not be clearly defined. It is widely believed, however, that C3bINA is responsible for cleavage of C3b into the C3c and C3d fragments (3). The purpose of this paper is to describe a method of isolation of C3bINA from human serum, its physical and chemical properties, its \(\beta 1H\) cofactor requirement and the products of its proteolytic action on C3b and C4b. One of the results obtained indicates that action of the C3bINA and its cofactor \(\beta 1H\) on soluble C3b does not generate the immunochemically defined fragments C3c and C3d.

Materials and Methods

Materials. The following materials were purchased from the sources indicated: serum was obtained from freshly drawn O positive human blood, purchased from Community Blood and Plasma Service, San Diego, Calif.; DEAE-cellulose (DE 52) from Whatman Chemicals, Div.

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\(^1\)Abbreviations used in this paper: C3bINA, C3b inactivator; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.
HUMAN C3b INACTIVATOR

W & R Balston, Maidstone, Kent, England; CNBr-Sepharose, Pharmacia Fine Chemicals, Piscataway, N. J.; guanidine HCl, ultrapure grade, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; dithiothreitol (DTT), Bio-Rad Laboratories, Richmond, Calif.; fuchsin-sulfite stain (Schiff reagent) from Harleco, Gibbstown, N. J.; and crystallized bovine serum albumin, Calbiochem, San Diego, Calif.

Purified Complement Components and Reagents. C1 (15), C2 (16), C4 (17), and C3 (12, 18) were isolated as described previously. C2 was used exclusively in the oxidized form (19). C1s was isolated by a modification (20) of the previously published method (21). β1H was purified from a pH 5.8 euglobulin precipitate of fresh human serum by gel filtration on BioGel A 1.5M (Bio-Rad Laboratories), DEAE-cellulose chromatography, hydroxyapatite chromatography, and finally by chromatography over an anti-IgG-Sepharose immune adsorbent. The product was free of C3bINA and gave a single precipitin line in immunoelectrophoresis versus anti-whole human serum. It also appeared to be homogeneous upon electrophoresis in sodium dodecyl sulfate (SDS) polyacrylamide gels. The cellular intermediate EAC423 was prepared by published methods (22) by using limiting C3 concentrations. Hemolytic assays with these cells utilized a C5-9 reagent prepared from serum immunochemically depleted of C3bINA. This depleted serum was then treated with 0.015 M hydrazine at 37°C for 45 min and dialyzed versus veronal buffer containing 0.15 × 10⁻³ M CaCl₂ and 1 × 10⁻³ M MgCl₂.

Preparation of Radiolabeled Proteins. Purified C3bINA, C3, and C4 were radiolabeled using bovine lactoperoxidase covalently coupled to Sepharose 4B as described by David and Reisfeld (23). Uptake of iodine was approximately 10% and the specific radioactivity of C3bINA was 0.37 ~Ci/μg, of C3 was 0.22 ~Ci/μg, and that of C4 was 0.6 ~Ci/μg.

Preparation of Immune Adsorbents. Anti-C3bINA, anti-IgG, and anti-β1H adsorbents were prepared by coupling the IgG fractions (40% ammonium sulfate precipitates) of goat antisera to CNBr-Sepharose utilizing the method of March et al. (24). The anti-C3bINA used in this study was raised in a goat by using C3bINA purified on an immune adsorbent prepared from rabbit anti-C3bINA. This original antiserum was a gift from Dr. Clark West.

Amino Acid Analysis. Amino acid analyses were performed on a Beckman 121-M amino acid analyzer (Beckman Instruments, Inc., Cedar Grove, N. J.). Samples were hydrolyzed for 24, 48, and 72 h with twice distilled, constant boiling 6 N HCl at 110°C in evacuated tubes. Tryptophan was determined after alkaline hydrolysis (25). Half-cystine and methionine were determined after performic acid oxidation.

Electrophoresis. Disk electrophoresis was performed by using the method of Davis (26) as modified by Clarke (27) in 6% polyacrylamide running gels and Tris-HCl-glycine buffer, pH 8.9. SDS polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (28) as reported previously (29). Denaturation analysis of stained gels was performed by using a Gilford recording spectrophotometer equipped with a linear transport unit. Gels stained with Coomassie brilliant Blue were scanned at 625 nm and those stained for carbohydrate by the periodic acid-Schiff technique (30) were scanned at 540 nm. Immunoelectrophoresis was performed in 1.2% agarose (Marine Colloids, Inc., Rockland, Maine) in barbital buffer, pH 8.6, ionic strength 0.05, according to the method of Scheidegger (31).

Results

Isolation of C3bINA. Fresh serum was brought to a final concentration of 0.02 M EDTA and 0.5 M NaCl by the addition of 0.2 M EDTA and solid NaCl. This solution was applied to a column of anti-C3bINA Sepharose (Fig. 1) and the column was washed with a buffer containing 0.5 M NaCl, 0.01 M PO₄, 0.02 M EDTA, pH 7.0, and 20% (wt/vol) sucrose. It was found that high concentrations of sucrose eluted a significant amount of protein which was probably bound to the polysaccharide backbone of Sepharose. After a second wash with buffer without sucrose, C3bINA was eluted with 2 M guanidine-Cl, containing 0.01 M sodium phosphate, 0.02 M EDTA, and 0.5 M NaCl. The material eluting with 2 M guanidine contained 47% of the applied C3bINA, but only 0.3% of the protein, a 142-fold purification (Table I).
FIG. 1. Affinity chromatography of serum on anti-C3bINA Sepharose. Fresh serum (110 ml) containing $1.9 \times 10^8$ cpm $^{125}$I-C3bINA (5.1 pM), 0.02 M EDTA, and 0.5 M NaCl was applied to a 5.6 x 6.2-cm column of anti-C3bINA Sepharose equilibrated with 0.01 M sodium phosphate, 0.002 M EDTA, and 0.5 M NaCl at pH 7.0. The column was then washed with 200 ml of equilibrating buffer, 200 ml of the same buffer containing 20% sucrose, and another 200 ml of equilibrating buffer. C3bINA was eluted with 200 ml of 2 M guanidine-HCl in the equilibrating buffer. C3bINA containing fractions were pooled as indicated by the bar.

| TABLE I | Purification and Yield of Human C3bINA Isolation |
|---------|-----------------------------------------------|
| Material | Volume | Total protein* | C3bINA Concentration† | Total C3bINA per step | Yield | Overall yield | Purification Per step | Total |
| Serum | 110 | 7,260 | 29 | 3.2 | — | — | 1 | 1 |
| Anti-C3bINA column pool | 9 | 24 | 145 | 1.5 | 47% | 47% | 142 | 142 |
| DEAE-cellulose pool | 0.5 | 0.63 | 1,000 | 0.5 | 33% | 16% | 12.7 | 1,810 |
| Anti-IgG column pool | 0.5 | 0.40 | 800 | 0.4 | 80% | 13% | 1.26 | 2,270 |

* Protein concentration determined by Folin analysis by using crystalline bovine serum albumin as a standard.
† C3bINA concentration was determined by radial immunodiffusion.

C3bINA eluted from the affinity column was dialyzed against 0.85% saline, 0.002 M EDTA at pH 7.2, concentrated by ultrafiltration and subsequently dialyzed against a solution of 0.008 M sodium phosphate, 0.005 M NaCl, pH 7.8, with a conductance at 21°C of 2.0 mmho/cm. This solution was applied to a column of DEAE-cellulose equilibrated with the same buffer (Fig. 2). The column was washed with three column volumes of starting buffer and C3bINA was subsequently eluted with a buffer of slightly higher sodium chloride concentration and a conductance of 4.1 mmho/cm. Although the yield of this step was only 33%, the protein eluted at 4.1 mmho was 85% C3bINA and the only detectable
contaminant was IgG. This protein was removed by passing the mixture over an anti-IgG Sepharose column. The resulting protein was homogeneous by all gel electrophoretic and immunological criteria tested. Fig. 3 demonstrates that upon disk gel electrophoresis at pH 8.9 a single protein band was observed upon staining with Coomassie Blue. The position of this band as well as the 125I-C3bINA peak corresponded to the position of enzymatic activity toward cell-bound C3b which was eluted from slices of a similar gel. Immunoelectrophoresis of purified C3bINA (Fig. 4) showed a single precipitin arc with anti-C3bINA. No contaminating antigens could be detected with anti-whole human serum. The protein migrated as a β-globulin.
Structural Properties: Molecular Weight, Subunits, Amino Acid, and Carbohydrate Content. Purified C3bINA was subjected to electrophoresis in 7% polyacrylamide gels containing SDS. Fig. 5 shows that before reduction of disulfide bonds the protein migrated as a single band and had an apparent mol wt of 73,000 daltons. Upon reduction, two polypeptide chains were separated having mol wt of 50,000 and 38,000 daltons. The mol wt of intact C3bINA was therefore determined to be 88,000 daltons. Both chains of this molecule were demonstrated to contain carbohydrate by periodic acid-Schiff staining of the separated chains on SDS gels. As shown in Table II, the ratios of the amount of protein-bound stain (Coomassie) correspond well to the molecular weight ratios for the respective polypeptide chains. The ratios of carbohydrate-bound stain in the two chains, however, indicates that the smaller chain contains the majority of the C3bINA-bound carbohydrates. A schematic drawing of the tertiary structure of the C3bINA molecule is shown in Fig. 6. Total hexose determinations by the phenol-sulfuric acid method gave a value of 7.5 μg hexose/100 μg C3bINA. Amino acid analysis for amino sugars yielded 3.2 μg of glucosamine/100 μg C3bINA. Since glucosamine does not react with the phenolsulfuric acid reagent, these values must be added to obtain the carbohydrate content of C3bINA, which is at least 10.7% not including neuraminic acid.

The amino acid composition of C3bINA is shown in Table III, calculated for a mol wt of 88,000 daltons. The composition suggests no unusual characteristics.

Normal Serum Concentration. The protein concentration of purified C3bINA was determined by amino acid analysis and by Folin analysis with bovine serum albumin as the reference protein. Purified C3bINA was then used to standardize radial immunodiffusion plates prepared with monospecific anti-
Fig. 5. SDS polyacrylamide gel electrophoresis of purified C3bINA. The subunit structure of C3bINA was examined by subjecting reduced and unreduced samples (70 μg) to electrophoresis through 7% polyacrylamide gels containing 0.1% SDS. Staining of the protein with Coomassie Blue demonstrated that the unreduced protein migrated as a single band which upon reduction dissociated into two polypeptide chains. A similar gel containing 21 μg C3bINA was stained for carbohydrate (right) by the periodic acid-Schiff technique.

C3bINA prepared in a goat. 20 normal sera, quantitated by radial immunodiffusion, contained an average of 34 ± 7 μg C3bINA/ml (mean ± 1 SD). The highest concentration found was 49 μg/ml and the lowest was 23 μg/ml.

Requirement of β1H for the Cleavage of Free C3b by C3bINA. Treatment of C3b with purified C3bINA at a concentration equivalent to that found in normal human serum for 60 min at 37°C had no effect on the structure of C3b (Fig. 7). C3b which had not been treated with anti-β1H Sepharose, however, was cleaved slowly by C3bINA due to the presence of a low level of β1H which usually contaminates preparations of C3. When C3b was incubated with C3bINA and β1H, both present in concentrations similar to those found in serum, the α'-chain of C3b (110,000 daltons) was cleaved into two fragments having mol wt of 67,000 and 40,000 daltons. These fragments will be referred to as α'-67 and α'-40.
TABLE II
C3bINA Polypeptide Chains: Comparison of Molecular Weight, Protein, and Carbohydrate Staining after SDS Polyacrylamide Gel Electrophoresis

| Chain | Mol wt | Proportion based on mol wt* | Proportion based on protein stain† | Proportion based on CHO stain‡ |
|-------|--------|-----------------------------|-----------------------------------|-------------------------------|
| α     | 50,000 | 57                          | 56                                 | 41                            |
| β     | 38,000 | 43                          | 44                                 | 59                            |

* Molecular weight determined by relative migration in 7% polyacrylamide gels containing SDS.
† Protein bands stained with Coomassie brilliant Blue R 250 and scanned as described in Methods.
‡ Gels stained for carbohydrate by the periodic acid-Schiff technique and scanned as described in Methods.

Neither of the fragments were released from C3b in the presence of SDS without reduction. Dissociation of α'-67 and α'-40 from the apparently intact β-chain required treatment with DTT. Cleavage in the presence of β1H was complete after 5 min and no additional cleavages occurred during 60 min of incubation (Fig. 7). The sum of the molecular weights of α'-67 and α'-40 equals the molecular weight of the α'-chain. No precursor of the two fragments other than the α'-chain could be detected in a time-course experiment utilizing 1/10th of the above indicated concentrations of C3bINA and β1H and extending over an observation period of 10–120 min at 37°C (lower right panel, Fig. 7).

For the formation of C3c and C3d from C3b, trypsin treatment was required in addition to treatment with C3bINA and β1H. C3b was first incubated with normal serum concentrations of C3bINA and β1H for 15 min at 37°C and then treated with 1 μg/ml trypsin for 30 min at 37°C. This treatment generated 27,000 and 140,000 dalton fragments which could be separated on SDS polyacrylamide gel electrophoresis without reduction. No fragments were released from unreduced C3b by trypsin under these conditions without prior treatment with C3bINA and β1H.

Requirement of β1H for the Cleavage of Free C4b by C3bINA. Native C4 is composed of three polypeptide chains referred to as α, β, and γ chains with mol wt of 93,000, 78,000, and 33,000 daltons, respectively (17). Treatment of C4 with C1s cleaves the C4a fragment from the α-chain, leaving the 86,000 mol wt α'-chain of C4b. Incubation of C4b with buffer, with C3bINA, or with β1H for 2 h at
TABLE III

Amino Acid Composition of Human C3bINA*

| Amino acid      | Residues/Molecule | Residues/1,000 Residues |
|-----------------|-------------------|-------------------------|
| Lysine          | 58                | 75                      |
| Histidine       | 17                | 21                      |
| Arginine        | 30                | 39                      |
| Aspartic acid   | 77                | 99                      |
| Threonine       | 45§               | 58§                     |
| Serine          | 61§               | 78§                     |
| Glutamic acid   | 87                | 111                     |
| Proline         | 32                | 41                      |
| Glycine         | 73                | 94                      |
| Alanine         | 45                | 57                      |
| Half-cystine    | 42§               | 54§                     |
| Valine          | 51¶               | 65¶                     |
| Methionine      | 11§               | 14§                     |
| Isoleucine      | 35¶               | 45¶                     |
| Leucine         | 43                | 55                      |
| Tyrosine        | 31§               | 40§                     |
| Phenylalanine   | 28                | 36                      |
| Tryptophan      | 14**              | 18**                    |
| Total           | 780               | 1,000                   |

* Reported as moles of amino acid per mole of protein, assuming a mol wt of 88,000 daltons.
† Average values from two replicates at each hydrolysis time (24, 48, and 72 h).
§ Extrapolated to zero time of hydrolysis.
¶ Determined after performic acid oxidation.
** Determined after base hydrolysis (25).

37°C did not alter the structure of the C4b molecule (Fig. 8). Cleavage of the α'-chain of C4b could, however, be accomplished by C3bINA in the presence of β1H. The lower right panel of Fig. 8 demonstrates that the simultaneous presence of these two proteins results in the complete loss of the α'-chain and the appearance of at least three fragments. These fragments have approximate mol wt of 49,000, 29,000, and 10–20,000 daltons. When a similar digest of C4b was subjected to gel electrophoresis in SDS without the reducing agent DTT, only the 49,000 mol wt fragment was released (Fig. 9). This suggests that the 49,000 mol wt fragment represents the nondisulfide bonded C4d portion of the molecule (5) and that the smaller fragments of the α'-chain are covalently linked to the β or γ chains of the molecule. Incubation of radiolabeled C4 with C3bINA and β1H did not alter the native three chain structure of C4, but some cleavage products appeared which probably originated from a small amount of C4b in the C4 (left panels, Fig. 8).

Discussion

This paper describes the first method for the purification of C3bINA as a homogeneous protein. It reports the C3bINA concentration in normal human serum to be 34 ± 7 μg/ml. It shows the enzyme to be composed to two nonidenti-
FIG. 7. Cleavage of C3b by C3bINA and β1H. Radiolabeled C3b (25 μg/ml) which had been treated with anti-β1H Sepharose was incubated with buffer or with 26 μg/ml C3bINA for 60 min at 37°C in a total vol of 20 μl. The mixture was denatured and reduced with an SDS-urea-DTT solution and applied to 7% SDS polyacrylamide gels. C3b treated with 500 μg/ml β1H under the same conditions gave a pattern identical to that shown for C3b alone. SDS gels of C3b incubated with both C3bINA (26 μg/ml) and β1H (500 μg/ml) for 60 min at 37°C, or with 1/10th these concentrations for 120 min at 37°C, are shown in the panels on the right. The mol wt of the α'-chain of C3b is 110,000 daltons and that of the β-chain is 75,000 daltons. The percent of the total recovered radioactivity found in the major fragments is written near each peak.

cal polypeptide chains which are linked by disulfide and noncovalent bonds. It delineates the chemical composition of the protein and the requirement of the cofactor β1H for its action on free C3b and C4b.

The purification of human C3bINA was greatly simplified by the use of immune adsorption on anti-C3bINA Sepharose. A single chromatographic separation on DEAE-cellulose of the eluate from the immune adsorption step rendered the protein 85% pure. Removal of contaminating IgG gave the product described in this study. The enzyme migrated as a single molecular species on polyacrylamide gel electrophoresis at pH 8.9 and C3bINA activity could be eluted from the region of the gel corresponding to the protein band. The protein appeared to be homogeneous and free of contaminating antigens upon immunoelectrophoresis by using an antiserum to whole human serum. Furthermore, a monospecific antiserum was raised in a goat after injection of highly purified C3bINA.

Polyacrylamide gel electrophoresis in the presence of SDS indicated that human C3bINA was composed of two polypeptide chains covalently linked by disulfide bonds. These chains had mol wt of 50,000 and 38,000 daltons and were
Fig. 8. The effect of C3bINA on the structure of C4 and C4b in the presence and absence of βH. $^{125}$I-C4 (7 × 10$^6$ cpm/μg) or $^{125}$I-C4b (prepared from $^{125}$I-C4 by incubation with C1a for 30 min at 37°C) were incubated with 0.8 μg C3bINA and where appropriate, 17 μg β1H for 2 h at 37°C in a total vol of 25 μl. Approximately 8 × 10$^4$ cpm were applied to each gel after denaturation and reduction in SDS-urea-DDT. Electrophoresis was performed in 7% polyacrylamide gels. The origin is at the left. The upper two panels show the results of incubations with C3bINA alone. Incubations with buffer or with β1H alone were essentially identical to these patterns. The percent of the total recovered radioactivity found in the major fragments is written near each peak.

Fig. 9. SDS polyacrylamide gel electrophoresis of an unreduced sample of $^{125}$I-C4b after treatment with C3bINA and β1H. The sample was prepared as described in Fig. 8, except that DTT was not used.

designated α and β, respectively, following the convention used with other complement proteins. Carbohydrate analyses indicated that the molecule contained 7.5% (wt/wt) hexose and 3.2% glucosamine, or at least 10.7% carbohydrate not including neuraminic acid. This carbohydrate appeared to be distributed
unevenly between the α and β chain, the smaller chain containing almost 60% of the carbohydrate.

The amino acid composition reported here is similar to that of other globulins, except for an above average content of half-cystine residues. While this value (42 residues per molecule or 5.4%) does not suggest any unusual properties, it may explain the stability of C3bINA to denaturation at pH 2.2 or by 2 M guanidine.

The physiological function of C3bINA has long been understood to be the cleavage of the C3b molecule which results in the abrogation of all known functions associated with C3b: the classical (32) and alternative (7, 8) pathway C5 convertases are inactivated, the immune adherence and opsonizing functions are lost (33), and the C3b-dependent positive feedback activity is destroyed (34). Concomitant with the loss of C3b activities, the reactivity with cellular C3d receptors (35) and with conglutinin (2) appears.

From previous studies it was concluded that proteolytic cleavage of the α'-chain of C3b (36, 37) by C3bINA splits the molecule into the immunochemically defined fragments C3c and C3d (38). Our results indicate that in complete absence of β1H, C3bINA does not cleave C3b in solution (Fig. 8). The previous observations of lack of β1H requirement for C3bINA action is attributable to the occurrence of small amounts of β1H in most preparations of highly purified C3. Removal of traces of β1H from the C3 used in this study was accomplished by immune adsorption with anti-β1H Sepharose. Action of C3bINA and β1H on C3b results in cleavage of the α'-chain into two fragments which do not dissociate without reduction. The mol wt of the two fragments are 67,000 and 40,000 daltons, respectively, both of which are larger than the mol wt of C3d (25,000 daltons) as isolated from aged or pathological sera (37). These observations indicate that an additional serum enzyme is required for the production of C3c and C3d. Trypsin can mimic the serum enzyme, but only subsequent to C3bINA and β1H action. The evidence at hand indicates that C3bINA and β1H generate only two α'-fragments without production of an intermediate cleavage product (85,000 daltons) as described by Gitlin et al. (36).

Some uncertainty has existed regarding the mode of regulation of the C4b fragment. Cooper (4) purified an enzyme from serum which cleaved and inactivated C4b and which he called C4b inactivator. Shiraishi and Stroud (5) reported that the C4b cleaving enzyme was identical with C3bINA, but that it required a high molecular weight serum cofactor to act on cell-bound C4b. Schultz and Arnold (39) and later Whaley and Ruddy (8) reported that highly purified C3bINA did not inactivate the immune adherence function of cell-bound C4b, which was originally described by Cooper (40).

In analogy to C3b regulation, the present study shows that C4b in solution was cleaved by C3bINA and β1H. Neither of the two proteins alone had any effect on C4b (Fig. 8). Cleavage of C4b produced two fragments of 150,000 and 49,000 daltons, respectively, which dissociated in the absence of reducing agents (Fig. 9). These findings are in good agreement with mol wt estimates made by gel filtration of C4b cleaved by serum enzymes (5) and with the mol wt of intact C4b (197,000) as previously described (17). SDS polyacrylamide gel electrophoretic analysis of the reduced fragments (Fig. 8) indicated that the C4d portion (49,000 daltons) came from the α'-chain of the C4b molecule. No cleavage of either the β or γ chains was detected. The α'-chain was cleaved during the 2-h
incubation into the 49,000-dalton C4d fragment, a 29,000-dalton fragment, and a fragment in the 10–20,000 mol wt range. Since at least three fragments of the α'-chain of C4b were generated, at least two proteolytic cleavages occurred. It seems unlikely that two cleavages would be produced by a proteolytic enzyme with the extremely restricted specificity C3bINA demonstrated with C3b. It is possible therefore that an unidentified second enzyme was present in the reaction mixtures. It remains to be determined which cleavage is effected by C3bINA and it is conceivable that, in analogy with C3b, the second enzyme causes the cleavage which generates the C4c and C4d fragments. Studies are currently underway to resolve this question and to identify the enzyme responsible for each cleavage.

Summary

The complement regulatory enzyme, C3b inactivator (C3bINA), has been purified from human serum by affinity chromatography on an anti-C3bINA Sepharose column. Subsequent chromatography on DEAE-cellulose and removal of IgG with anti-IgG Sepharose resulted in a product which was found to be homogeneous by polyacrylamide gel electrophoresis at pH 8.9 and by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecule is composed of two disulfide bonded polypeptide chains with mol wt of 50,000 and 38,000 daltons. Human C3bINA was found to be a glycoprotein containing at least 10.7% carbohydrate and to have a normal serum concentration of 34 ± 7 μg/ml (mean ± 1 SD). Highly purified C3bINA cleaved neither free C3b nor free C4b if trace amounts of contaminating β1H were removed from these proteins with anti-β1H Sepharose. However, in the presence of highly purified β1H and C3bINA, both C3b and C4b were cleaved. Incubation of native C3 or C4 with C3bINA and β1H had no effect on their structure. The action of C3bINA and β1H on C3b produced two fragments of the α'-chain which did not dissociate without reduction of the molecule. These fragments have mol wt of 67,000 and 40,000 daltons. The action of C3bINA and β1H on C4b resulted in cleavage of the α'-chain giving rise to the 150,000-dalton C4c and the 49,000-dalton C4d fragments, which dissociated without reduction. To produce from C3b the immunochemically defined C3c and C3d fragments, the action of an additional serum enzyme appears to be required, the effect of which can be mimicked by trypsin.

Received for publication 1 April 1977.

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