# ISOLATION OF A FRAGMENT (C3a) OF THE THIRD COMPONENT OF HUMAN COMPLEMENT CONTAINING ANAPHYLATOXIN AND CHEMOTACTIC ACTIVITY AND DESCRIPTION OF AN ANAPHYLATOXIN INACTIVATOR OF HUMAN SERUM*,‡

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The third component of human complement, C3, has been shown to be cleaved by its activating enzyme C3 convertase (1) and by the C3 inactivator complex (2) into two fragments called C3a and C3b (3, 1). The C3a fragment which is the smaller of the two pieces was found to have anaphylatoxin activity (3, 4). Streptokinase-activated plasminogen also was found to cleave a small fragment off the C3 molecule which, however, was different from C3a in that it exhibited leukocyte chemotactic activity, but no anaphylatoxin activity (5, 6).

The present study was initiated (*a*) to investigate cleavage of the native C3 molecule by a proteolytic enzyme with defined and restricted bond specificity, such as trypsin; (*b*) to isolate and characterize the fragments obtained; and, if possible, (*c*) to relate biological activities to defined fragments of the C3 molecule. It was found that trypsin cleaves the C3 molecule into at least four distinct fragments, two of which closely resemble C3a and C3b, and that both the anaphylatoxin and the chemotactic activities are resident in the C3a, although in different regions of this fragment. The C3a fragment could be isolated in amounts sufficient for physical and chemical characterization, and an antiserum capable of inhibiting its biological activity could be produced. In the course of this work, an inactivator of anaphylatoxin was found in human serum fractions and evidence was obtained, suggesting that it destroys anaphylatoxin activity by an enzymatic mechanism. Part of the results contained in this communication were presented earlier in abridged form (7, 8).

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## *Materials and Methods*

*Highly Purified C3 of Human Serum.*—The protein was isolated as described previously (9). Purity was analyzed by Ouchterlony test and immunoelectrophoresis using a potent anti-whole human serum. Each preparation was also analyzed by disc electrophoresis both at pH 8.7 (10) and pH 4.5 (11). Preparations which contained detectable impurities were omitted. To obtain radioactive C3, the protein was labeled with $^{125}I$ or $^{131}I$ by the chloramine T method (12). The specific radioactivity varied between 50,000 and 100,000 cpm/$\mu$g protein.

*Antisera.*—Anti-human C3 was produced by injecting rabbits four times subcutaneously with 0.5 mg of the purified protein in complete Freund's adjuvant at 1 wk intervals. Anti-C3a was produced by injecting 30 $\mu$g of isolated C3a in complete Freund's adjuvant into the popliteal lymph nodes of rabbits and 3 wk later the same material intramuscularly. The antisera were monospecific for C3 when tested against whole human serum. Antisera to whole human serum were purchased from Behringwerke A. G., Marburg-Lahn, West Germany. A sample of antiserum to human $\alpha_{2D}$ was kindly furnished by Dr. Clark West, Cincinnati, Ohio.

*Enzymes and Enzyme Inhibitors.*—Crystalline trypsin was purchased from Worthington Biochemical Corp., Freehold, N. J.; purified plasminogen (13) and thrombin (14) were kindly made available by Dr. Fletcher Taylor of the University of Pennsylvania. Streptokinase was purchased from American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y.; for activation, 500 $\mu$g plasminogen was treated at pH 7.5 at 37°C for 30 min with 1500 units of streptokinase. C3 convertase ($\overline{C4,2}$) was generated as described previously (1). C3 inactivator complex (C3IC) was obtained by incubating partially purified C3 serum proinactivator with purified cobra factor in the presence of $Mg^{++}$ (2). Soybean trypsin inhibitor (SBTI), Lot No. 9150, five times crystallized, was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, and diisopropylfluorophosphate (DFP) from Boots Pure Drug Co., Ltd., Nottingham, England.

*Method of Formation and Isolation of C3a and C3b.*—Three different procedures were used for the isolation of C3a: Sephadex filtration, preparative gel electrophoresis, and free flow electrophoresis. The sample applied contained approximately 50 mg of a mixture of purified, unlabeled C3 and of $^{125}I$-C3 which had been treated at a concentration of 1 mg/ml with 1% trypsin (w/w) for 60 sec at 20°C and pH 7.6. The reaction was stopped by addition of SBTI in an amount twice that of trypsin present. The sample was then acidified with 1.0 N HCl to pH 3.5. Since some of the C3b fragment precipitates upon acidification, the sample was cleared by brief centrifugation and then concentrated to approximately 5 ml using an Amicon pressure filtration device and the ultrafilter UM-05. After separation of the material, the C3a fragment was detected by analyzing the collected fractions for radioactivity, and fractions containing C3a were pooled and concentrated as outlined above. Approximately 2 mg C3a were obtained from 50 mg C3.

Sephadex filtration was carried out using a 90 × 2.5 cm column of Sephadex G-100 which was equilibrated with 0.15 M acetate buffered sodium chloride, pH 3.6. This buffer consisted of nine parts 0.01 M acetic acid, one part 0.01 M sodium acetate, and 8.78 g of NaCl/liter. Five ml of sample was layered between the top of the column and the supernatant buffer. Filtration was carried out at 4°C with a flow rate of 15 ml/hr and fractions of 3.5 ml were collected.

Preparative gel electrophoresis was performed in a Buchler apparatus using a 2.5 cm separating column of 6% polyacrylamide gel concentration and buffer of pH 4.5 as described by Reisfeld et al. (11). A current of 100 ma was applied, the flow rate was adjusted to 10 ml per hour and fractions of 2.5 ml were collected. Under these conditions C3a was completely eluted after 6 hr.

Free flow electrophoresis was carried out in a Hannig apparatus (Electrophoretic separator

Model FF, Brinkmann Instruments, Westbury, New York). The electrode buffer (pH 4.0, conductance 7.3 millimhos/cm) contained 133 g of sodium acetate and 280 ml of concentrated acetic acid in 10 liters of distilled water. The chamber buffer was obtained by dilution of the electrode buffer with distilled water to adjust the conductance to 2.2 millimhos/cm. The sample was applied into the port next to the anode, the buffer flow in the chamber was adjusted to 120 ml/hr and a current of 200 ma was used which corresponded to 1880 v across the chamber.

The C3b fragment was separated from C3a and small amounts of trypsin and SBTI by Sephadex filtration of trypsin treated C3 (1% trypsin, w/w, 120 sec, 20°C, pH 7.6) in 0.15 M acetate buffered sodium chloride, pH 4.0. C3b appeared in the exclusion volume of the column.

*Method of Formation and Purification of C3c and C3d.*—10 mg of C3 (1 mg/ml) were treated with 2% trypsin (w/w) in Veronal buffer, pH 7.6, for 60 min at 37°C. The reaction was stopped by addition of SBTI in two-fold excess over trypsin. The trypsin-treated C3 was concentrated to 2 ml and chromatographed on a column of Sephadex G-200 (97 × 1.5 cm), equilibrated with 0.15 M Veronal-buffered sodium chloride, pH 7.6. The distribution of C3c and C3d in column fractions was detected by serial Ouchterlony analysis on microscopic slides using an antiserum to native C3 and to $\alpha$ 2D.

*Analytical Polyacrylamide Gel Electrophoresis.*—This was carried out according to Reisfeld (11) using 6% resolving gel and $\beta$-alanine-acetic acid buffer, pH 4.5, for demonstration of C3a and Tris-HCl buffer, pH 8.7 (10) for the demonstration of C3b, C3c, and C3d.

*Molecular Weight Determinations.*—Calculations were made from separate determinations of sedimentation ($s$) and diffusion coefficients (D), assuming a partial specific volume of 0.73 for all proteins. This value was derived from the amino acid compositions of both C3 and C3a (15). D was estimated according to Andrews (16) employing columns of Sephadex G-200 equilibrated with 0.15 M Tris-HCl buffer, pH 8.2. As reference substances were used, equine cytochrome C ($13 \times 10^{-7}$ cm²/sec), hemoglobin ($6.8 \times 10^{-7}$ cm²/sec), $^{131}$I $\gamma$G-globulin ($3.8 \times 10^{-7}$ cm²/sec) and $^{131}$I thyroglobulin ($2.5 \times 10^{-7}$ cm²/sec), s was estimated by ultracentrifugation in a 3.85–15.5% linear sucrose density gradient in an L2 Spinco machine using an SW39 or SW50 rotor. 0.1 M Tris-HCl buffer, pH 8.2, was used for most experiments; for the analysis of C3a, a calibrated Sephadex G-100 column was used, equilibrated with acetate-buffered sodium chloride of pH 3.6. Reference substances were $^{131}$I $\gamma$G-globulin (7S), hemoglobin (4.6S), not used in experiments where a low pH was employed, and equine cytochrome C (1.7S).

The mol wts of C3 and C3a were also determined according to Archibald by approach to sedimentation equilibrium using the method described by Trautman (17). C3 was analyzed in phosphate buffer, pH 7.0, T/2 (ionic strength) = 0.1, at a concentration of 7.8 mg/ml at 15,220 and at 8,766 rpm and 20°C. C3a was examined at 5.8 mg/ml in 0.15 M acetate-buffered sodium chloride, pH 3.6, and 20°C at 52,640 rpm and at 39,460 rpm. The resulting $c_m - c^\circ$ versus $q_m$ plot from which the mol wt of C3a was calculated is shown in Fig. 1.

*Demonstration of Biological Activity.*—The ability of fragments to contract the guinea pig ileum and to cause changes in vascular permeability was tested as described earlier (4). Chemotactic activity was determined with rabbit polymorphonuclear leukocytes according to published methods (6).

*Purification of an Anaphylatoxin Inactivator from Human Serum.*—Human blood was allowed to clot for 4 hr and the clot was removed by centrifugation. To remove the euglobulins, the serum was dialyzed for 48 hr against 10 liters of $8 \times 10^{-3}$ M $Na_3$ EDTA[1] of pH 5.4, having a conductance of 1.25 millimhos/cm. 10 ml of the supernatant pseudoglobulin fraction was subjected to Pevikon block electrophoresis in barbital buffer, pH 8.6, T/2 = 0.05, for 24 hr

[1] Ethylenediamenetetraacetate.

at 4°C using a potential gradient of 4 v/cm. Fractions containing anaphylatoxin inactivator activity were pooled, concentrated to approximately 2 ml and applied to a Sephadex G-200 column (97 × 1.5 cm) equilibrated with 0.15 M Tris-HCl buffer, pH 8.0. The flow rate was adjusted to 8 ml/hr and fractions of 1.7 ml were collected and tested for inhibitory activity. The active fractions were pooled, concentrated, and subjected to analytical polyacrylamide gel electrophoresis in 6% gels and at pH 8.7. Several gels were utilized; two were stained for proteins. The others were left unstained and cut into 2 mm segments, each of which were eluted with veronal-buffered sodium chloride, pH 7.6, and tested for inactivating activity.

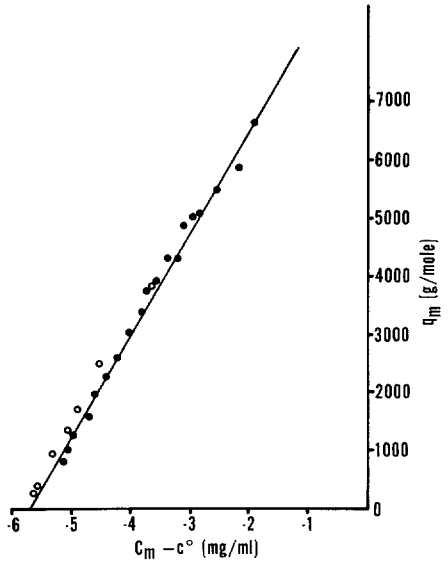


FIG. 1. $c_m - c^\circ$ vs. $q_m$ plot for the mol wt determination of isolated C3a by the approach to sedimentation equilibrium method. The protein was analyzed at 5.8 mg/ml in 0.15 M acetate-buffered sodium chloride, pH 3.6, at 20°C and 39,460 rpm (solid circles) and 52,640 rpm (open circles). M $(1 - \bar{v})$ = slope of line. By least square method $y = -1761.8\ x + 10045.6$. ($\bar{v}$ = partial specific volume).

*Anaphylatoxin Inactivator Assay.*—10 μg of C3a were allowed to react with 0.1 ml of various column fractions for 4 min at pH 7.0 and 20°C. C5 derived anaphylatoxin was obtained by trypsin treatment of isolated C5 as described previously (4). 150 μg of treated but unseparated-C5 was then allowed to react with 0.1 ml of the column fractions as above. The reaction mixtures were tested for anaphylatoxin activity on sensitized guinea pig ileum as described else where (4).

*Nomenclature.*—Symbols and terms used in this publication were recommended by the World Health Organization Committee on Complement Nomenclature, Boston, 1968. The recommended changes of the previously used nomenclature (18) include deletion of the prime from the complement symbol (i.e., C instead of C′) and deletion of "a" as indication for an activated complement component. Enzyme activity may be indicated by placing a bar above the numeral which refers to the component in which the activity resides. Thus, C3 convertase may be denoted $C\overline{4,2}$. Fragments of components resulting from cleavage by enzymes are de-

noted with a small letter, e.g., C3a, C3b, C3c, and C3d. Inactivated components or inactivated products of components may be designated with the small letter "i".

## RESULTS

*I. Cleavage of Native C3 into Four Fragments by Trypsin.*—

*A. Demonstration of the C3 fragments:* Treatment of native C3 with trypsin for 60 sec at room temperature resulted in an increase of electrophoretic mobility and in complete loss of hemolytic activity. Immunoelectrophoresis detected a major product which resembled the previously described C3i (19) (Fig. 2, top). Disc electrophoresis at pH 4.5 revealed a second, minor product which migrated rapidly toward the cathode (Fig. 2, bottom). The minor fragment was detected

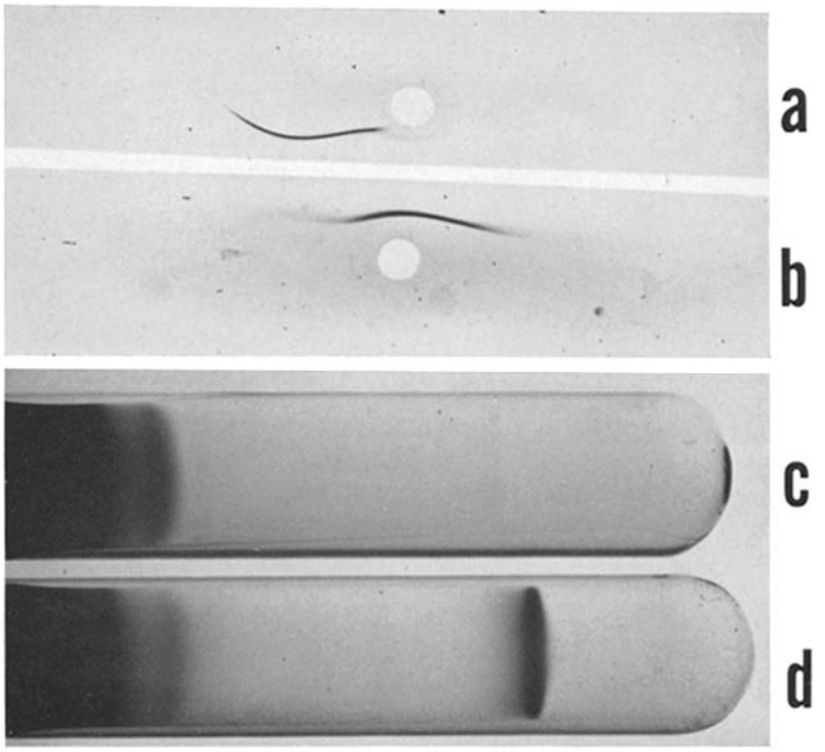


FIG. 2. Demonstration of C3a by acid polyacrylamide gel electrophoresis following treatment of C3 with trypsin (1%, w/w, 60 sec, 20°C, pH 7.6). Upper panel: immunoelectrophoresis of (*a*) native C3 and (*b*) trypsin treated C3. Anode was at the right; pattern was developed with rabbit anti-human C3. Lower panel: electrophoresis in 6% polyacrylamide gel at pH 4.5. (*c*) 200 μg of native C3, (*d*) 200 μg of trypsin treated C3. The cathode was at the right. The lower gel (*d*) shows C3a as a cathodically migrating protein band. The dark areas near the anodal end of the gels represent native C3 and C3b, respectively. Identical results were obtained after treatment of C3 with C3 convertase.

without exception in replicate experiments, but was never observed when native C3 was subjected to disc electrophoresis. It will be referred to as C3a, and the product resembling C3i will be called C3b.

Treatment of C3 with trypsin for 1 hr at 37°C resulted in degradation of the initially liberated C3a fragment and in cleavage of the C3b fragment into two pieces, C3c and C3d, which were readily demonstrable by immunoelectrophoresis (Fig. 3).

*B. Isolation of the C3 fragments:* Utilizing $^{125}$I-labeled, isolated C3 and limited trypsin treatment (60 sec, 20°C), C3a was separated from C3b at acid pH either by filtration on Sephadex G-100 (Fig. 4), by preparative polyacrylamide gel electrophoresis (Fig. 5), or by preparative free flow electrophoresis in a Hannig apparatus (not shown). C3a purified by these methods appeared as a single disc when analyzed by analytical polyacrylamide gel electrophoresis at pH 4.5, as shown in Fig. 5. Dissociation of the a-fragment from cleaved C3 required acid pH; it did not occur at pH 7.

After prolonged trypsin treatment of native C3 (60 min, 37°C), the c- and the d-fragment were separated by filtration on Sephadex G-200, as shown in Fig. 6.

*C. Physicochemical and immunologic characterization of the C3 fragments:* Values for sedimentation and diffusion coefficients and for the mol wts of the fragments are listed in Table I. For comparison, respective parameters for native C3 are also listed. The mol wt data are in good agreement with the relative yield of the fragments, which for the a-, b-, c- and d-fragments was found to be 5%, 95%, 60%, and 14%, respectively. Fission of the b-fragment was invariably accompanied by liberation of dialyzable peptides which accounted for approximately 20% of the starting material. The mol wts of C3 and C3a were also determined by the Archibald method. The discrepancy in values obtained for native C3 by different methods, although unexplained, is presumed to be methodological in nature. Fig. 7 shows schlieren patterns of C3a which were photographically recorded during analytical ultracentrifugation.

The electrophoretic mobility of the small C3a fragment differed strikingly from that of any protein of serum. As illustrated in Fig. 8, C3a migrated on cellulose acetate strips at pH 8.6 toward the cathode, whereas the $\gamma$-globulin fraction of a reference serum moved from the origin toward the anode. In contrast, C3c and C3d differed little in electrophoretic behavior and resembled C3b (Fig. 3). C3d appears to be electrophoretically heterogeneous. Further treatment with trypsin for 15 min converted C3a to a product which at pH 8.6 was negatively charged and migrated as a broad zone with the electrophoretic mobility of the $\beta$-globulin fraction (Fig. 8).

When isolated C3a was analyzed with anti-C3 by capillary precipitin test, turbidity developed within 10 min and a flocculent precipitate became visible after several hr. A definite precipitin line could be seen upon analysis of C3a with either anti-C3 or anti-C3a in Ouchterlony plates (Fig. 9). The precipitin

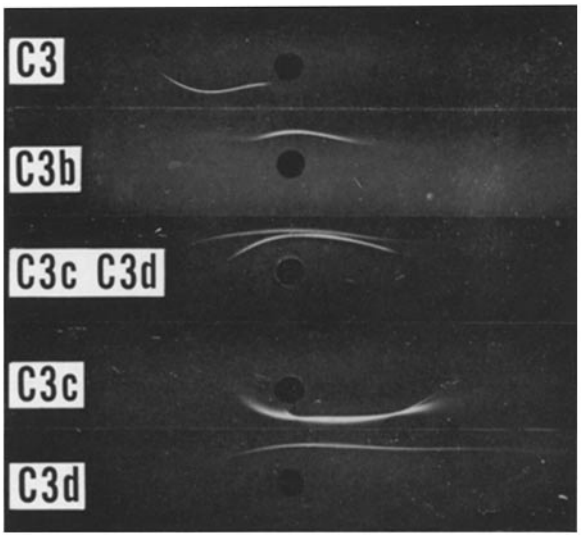


FIG. 3. Immunoelectrophoretic demonstration of fragments C3b, C3c, and C3d. C3b was obtained by treatment of C3 with 1% (w/w) trypsin for 60 sec at 20°C (C3a not detectable). C3c and C3d were obtained by treatment of C3 with 2% trypsin (w/w) for 60 min at 37°C. These fragments were separated by Sephadex G-200 filtration. The anode was to the right; patterns were developed with rabbit anti-human C3.

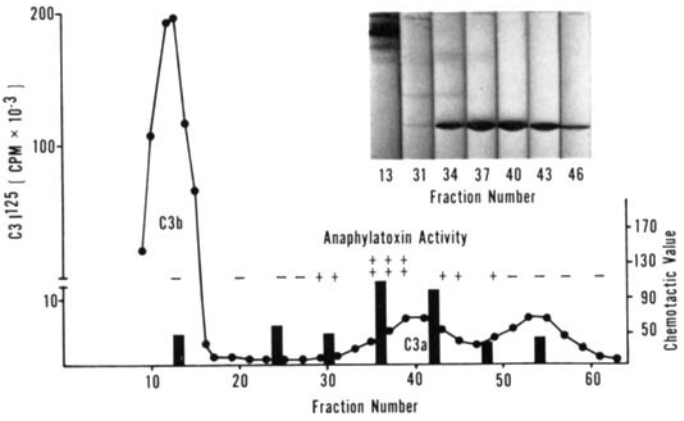


FIG. 4. Isolation of C3a by Sephadex G-100 filtration and correlation of the distribution of C3a with anaphylatoxin and chemotactic activity. 30 mg purified C3 containing a small amount of $^{125}$I-C3 was applied to the column after treatment with 1% trypsin (w/w) for 60 sec at 20°C. The column was equilibrated with 0.15 M acetate buffered sodium chloride, pH 3.6. Solid line shows distribution of radioactivity in fractions, bars indicate chemotactic activity, the symbol + shows anaphylatoxin activity. C3b, the first peak, emerged with the exclusion fraction; C3a accounted for the second peak as shown by acid disc electrophoresis of fraction samples (insert: discs in fractions 31–46 in lower part of gels constitute C3a). The third peak was caused by free $^{125}$I and a negligible amount of small peptides.

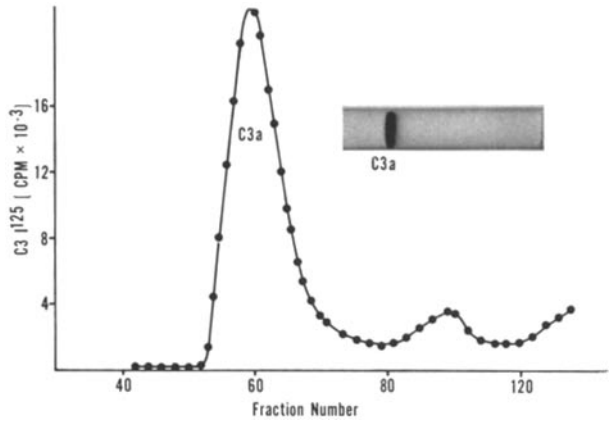


FIG. 5. Isolation of C3a by preparative polyacrylamide gel electrophoresis using a Buchler column. 30 mg purified C3 containing trace amounts of $^{125}$I-C3 was applied to the column after treatment with 1% trypsin (w/w) for 60 sec at 20°C. The resolution gel was 2.5 cm long, the gel concentration 6%, the pH 4.5, the current 100 ma, and the time of electrophoresis 15 hr. The cathode was located at the bottom of the column corresponding to the left side of the graph. C3a emerged after approximately 6 hr, C3b was still inside the gel at the end of the experiment. Fractions of the major peak were pooled, concentrated, and the protein analyzed by acid disc electrophoresis (insert).

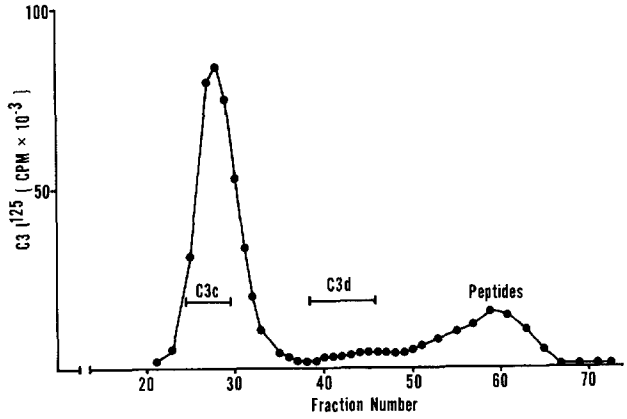


FIG. 6. Separation of C3c and C3d by filtration of Sephadex G-200. 10 mg C3 containing a small amount of $^{125}$I-C3 was applied after treatment with 2% trypsin (w/w) for 60 min at 37°C. The column was equilibrated with 0.15 M veronal-buffered sodium chloride, pH 7.6. The distributions of the c- and d-fragment were detected by antisera to C3 and $\alpha_{2D}$ (not shown). To avoid cross contamination, fractions were pooled as indicated.

TABLE I

*Molecular Parameters of C3 and C3 Fragments*

| Fragment | *s* | D | Molecular weight | |
|---|---|---|---|---|
| | | | Calculated from *s* and D | Archibald method |
| Native C3 | 9.5 | 3.6* | 235,000 | 180,000 |
| C3a | 1.1 | | 8,700‡ | 7,000 |
| C3b | 9.0§ | 3.6* | 223,000 | |
| C3c | 6.7§ | 4.0* | 151,000 | |
| C3d | 2.6§ | 8.6* | 27,000 | |

* Sephadex G-200 filtration.

‡ Determined with calibrated Sephadex G-100 column.

§ Sucrose density gradient ultracentrifugation.

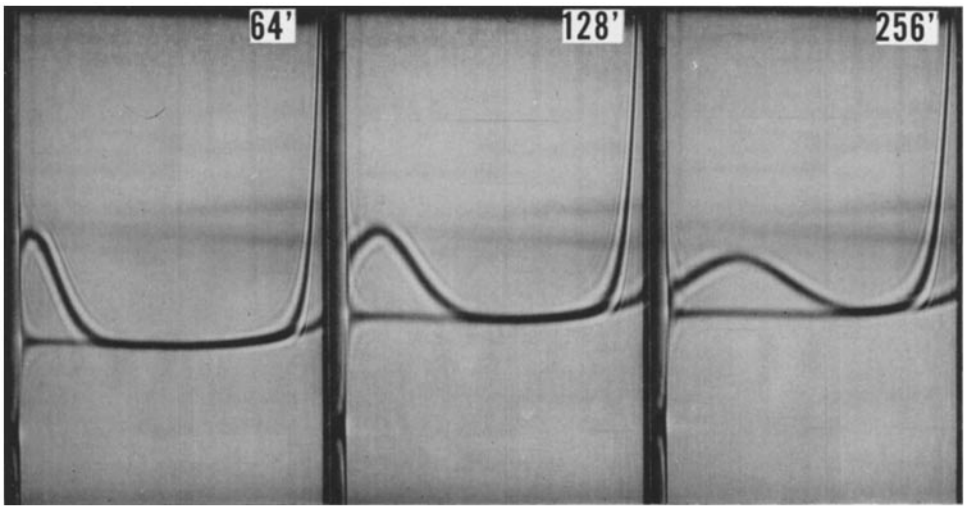


FIG. 7. Schlieren patterns of isolated C3a obtained during analytical ultracentrifugation. Protein concentration, 5.8 mg/ml; solvent, 0.15 M acetate-buffered sodium chloride, pH 3.6; temperature, 20°C; phase-plate angle at 64 min, 50°, at 128 and 256 min, 45°; speed, 52,640 rpm.

patterns indicated that C3a is antigenically distinct from C3b[2] and that both antigenic entities are recognizable in the native C3 molecule. They also showed that the antigenic deficiency of C3b relative to native C3 is not entirely ac-

[2] Unless C3b is passed over Sephadex at acid pH (4.0), it is loosely complexed with C3a and yields a positive reaction with anti-C3a. A lower pH should be avoided, as it destroys the reactivity of C3b with antibody.

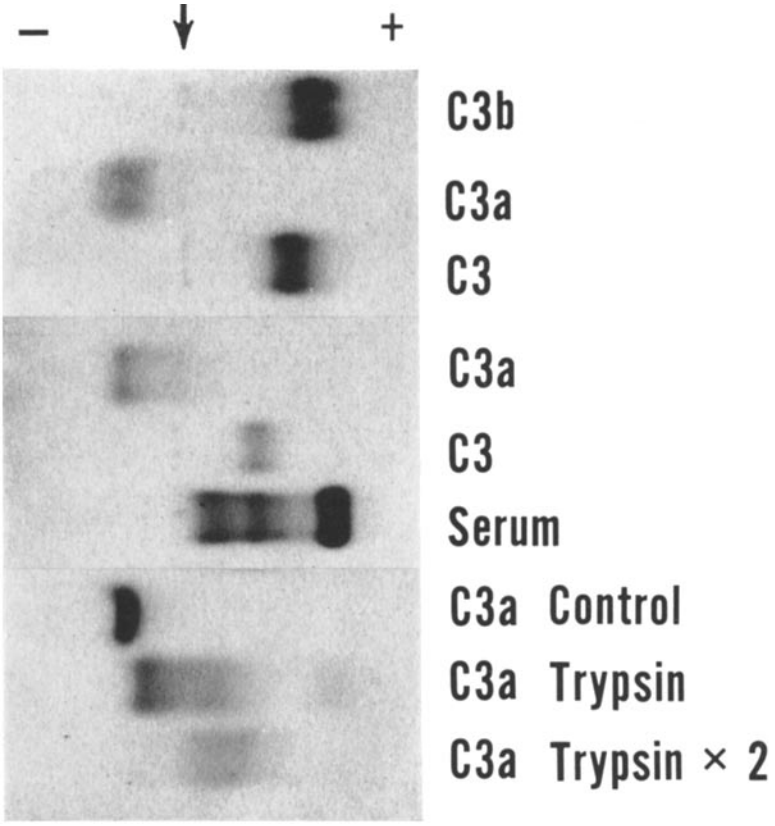


FIG. 8. Demonstration of basic character of isolated C3a by electrophoresis on cellulose acetate at pH 8.6. The figure is a composite of three electrophoretic strips which were subjected to electrophoresis under identical conditions on different days. The upper strip shows the cathodal migration of C3a relative to C3 and C3b. The strip in the middle shows the electrophoretic behavior of C3a relative to whole human serum and C3. The lower strip illustrates the effect of trypsin (15 min, 37°C, 4% and 8% [w/w]) on the mobility of C3a. Three different preparations of C3a were used. The arrow indicates the site of application.

counted for by the lack of the small a-fragment. This was also shown in other experiments in which absorption of anti-C3 with purified C3a did not abolish the characteristic spurring of the C3 precipitin line over the C3b line. Analysis of the purified fragments C3c and C3d by Ouchterlony test using an anti-C3 antiserum showed that both fragments were antigenically distinct, since the precipitin pattern gave the reaction of non-identity (Fig. 10). Further, C3c gave a reaction of identity with $\beta_{1A}$-globulin isolated from aged serum and C3d (but not C3c) reacted with antiserum to $\alpha_{2D}$.

*II. Studies of the Biological Activities of the C3a Fragment.*—

*A. Anaphylatoxin and chemotactic activity of C3a produced by trypsin:* C3 (1 mg/ml) treated with trypsin for various periods of time[3] was tested for smooth

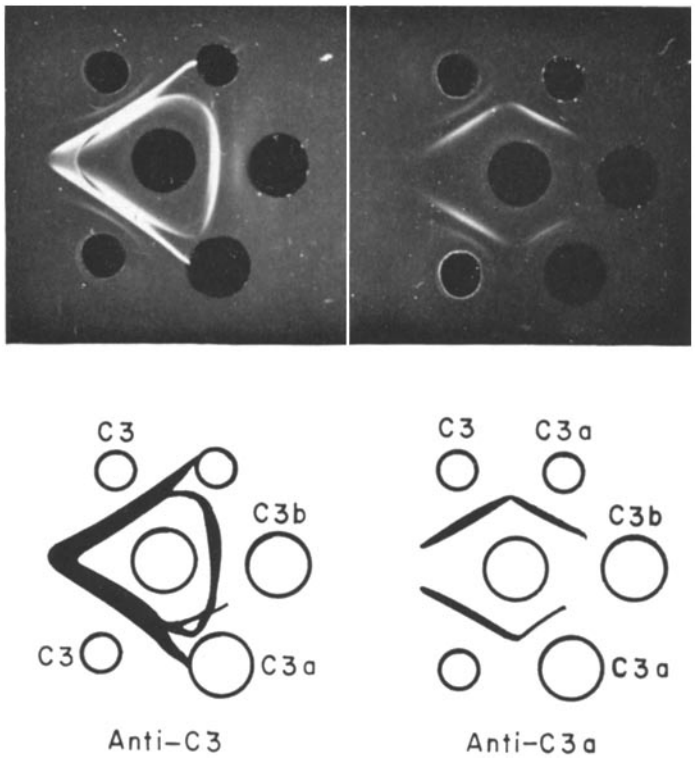


FIG. 9. Demonstration of antigenic difference between C3a and C3b by Ouchterlony analysis. The drawings below the photographs were made according to visual inspection of the agar plates and emphasize the essential phenomena, which cannot be seen clearly in the photographic record. The precipitin line resulting from C3a and anti-C3 crossed the C3b-anti-C3 line. Anti-C3a reacted with C3a and C3 and both lines fused completely, but it did not react with C3b. The identity of the lines near the C3 wells is unknown.

muscle contracting activity. Definite activity was reproducibly demonstrated and, as listed in Table II, maximal activity was observed after 1 min at 20°C. In other experiments, using C3 in concentrations of 10 mg/ml, activity was seen as early as 6 sec after addition of trypsin. The active principle was itself sensitive to the effects of trypsin and was no longer detectable after 5 min of

[3] Following treatment with trypsin, SBTI was added and the samples were then acidified with 0.1 N HCl to pH 3.5.

trypsin treatment. The active material also produced increased vascular permeability in guinea pig and rabbit skin, and both biological effects were inhibitable by anti-histamine. In addition, trypsin treated C3 caused histamine release from isolated rat peritoneal mast cells.

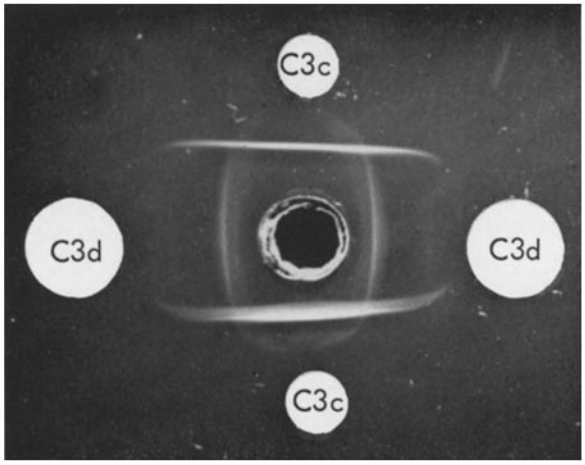


FIG. 10. Ouchterlony analysis showing antigenic distinctness of C3c and C3d. Center well: anti-C3.

TABLE II

*Generation of Anaphylatoxin and Chemotactic Activity from C3 by Trypsin as a Function of Time at 20°C*

| Time of treatment* with trypsin | Anaphylatoxin activity | Chemotactic value |
|---|---|---|
| 30 sec | +++ | 63 |
| 1 min | ++++ | 110 |
| 2 " | +++ | 103 |
| 5 " | + | 98 |
| 10 " | − | 115 |
| 30 " | − | 112 |

* 1 mg C3 in 1 ml 0.15 M Veronal-buffered sodium chloride, pH 7.6, was treated with 1% trypsin (w/w). Reaction was stopped by addition of SBTI.

Chemotactic activity for polymorphonuclear leukocytes also appeared upon treatment of purified C3 with trypsin (Table II). The development of chemotactic activity followed approximately the time course of generation of anaphylatoxin. Maximal activity was observed after 1 min at 20°C, coincident with complete conversion and loss of hemolytic activity of C3 and with maximal liberation of the C3a fragment. Unlike anaphylatoxin, chemotactic activity was

resistant to further trypsin treatment up to 1 hr at 20°C, at which time it gradually decreased.

Filtration of trypsin treated $^{125}$I-C3 containing anaphylatoxin and chemotactic activity on a Sephadex G-100 column showed that both activities had a

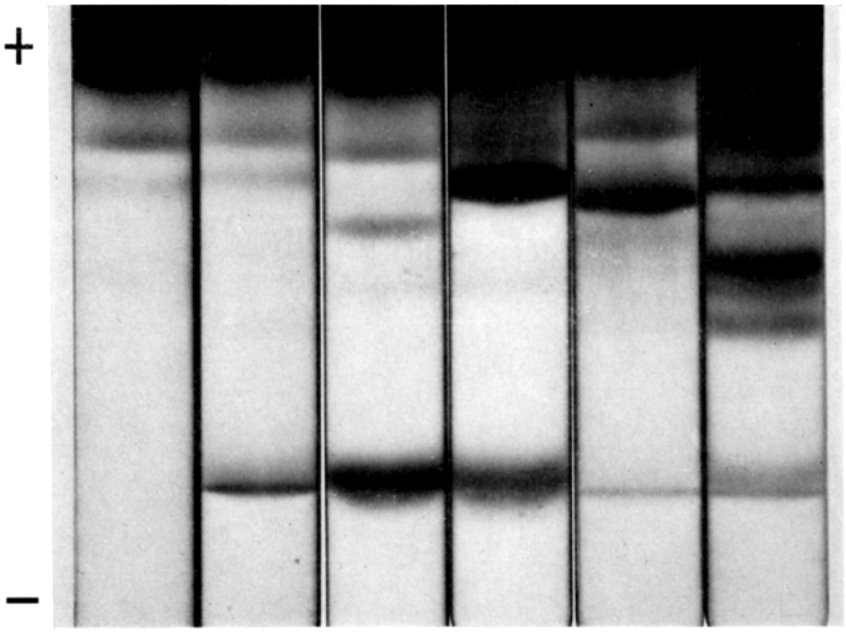


FIG. 11. Acid polyacrylamide gel electrophoresis demonstrating formation of basic fragment by treatment of native C3 with various enzymes. 150–200 μg of C3 were applied to each gel; the gel concentration was 6% and the pH was 4.5. Contr., untreated C3; Try., 1% trypsin (w/w), 30 seconds, 20°C; Conv., C3 convertase, 30 min, 37°C; C3IC, C3 inactivator complex, 30 min, 37°C; Plasm., plasmin, 100 μg, 30 min, 37°C; Throm., thrombin, 50 N.I.H.* units, 30 min, 37°C. All controls (not shown) lacking enzyme appeared like untreated C3, and all enzyme samples lacking C3 gave patterns which accounted for the additional protein bands seen in the treated samples but not in the untreated control, except the band labeled C3a.

* National Institutes of Health.

comparable distribution and that they emerged together with C3a (Fig. 4). This finding was interpreted to indicate that the C3a fragment liberated by 1 min of treatment with trypsin at 20°C is endowed with anaphylatoxin as well as with chemotactic activity. Between one and three micrograms of isolated C3a were sufficient to produce detectable anaphylatoxin activity. Iden-

*B. Comparative analysis of C3a (trypsin) and C3a produced by other enzymes:* tical samples of purified C3 were incubated with the following enzymes under specified conditions: C3 convertase, C3 inactivator complex, streptokinase activated plasmin, thrombin, and trypsin. The treated samples were then analyzed by disc electrophoresis at pH 4.5. As seen in Fig. 11, in each instance a basic fragment was liberated and all basic fragments had similar gel electrophoretic properties and resembled C3a (trypsin).

All of the treated samples, except that containing thrombin, had smooth

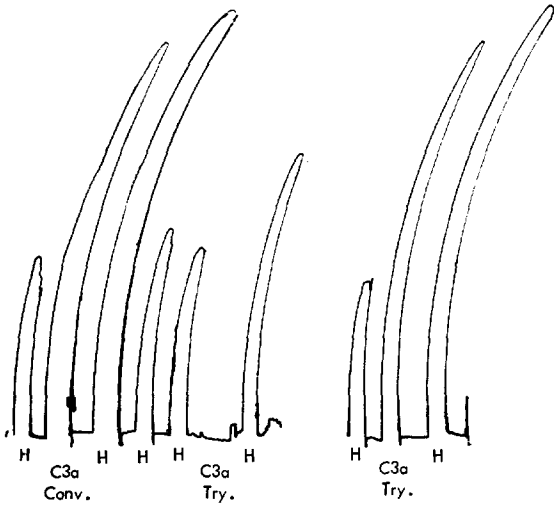


FIG. 12. Identity of biological activity of C3a produced by trypsin and by C3 convertase, demonstrated by unresponsiveness of a segment of guinea pig ileum to C3a (trypsin) after desensitization with C3a (C3 convertase). That the trypsin-produced C3a had smooth muscle contracting activity is shown by effect on a separate segment of guinea pig ileum. H indicates the effect of histamine.

muscle contracting activity. Application of C3a, liberated by the action of C3 convertase, to the guinea pig ileum produced tachyphylaxis and desensitized the muscle to the effect of C3a (trypsin) (Fig. 12), indicating identical biologic specificity of the two fragments. A desensitized muscle, however, retained full reactivity to C5 derived anaphylatoxin. In view of the finding of chemotactic activity in C3a (trypsin), the C3a fragments produced by C3 convertase and by the C3 inactivator complex were isolated and tested. Both preparations were found to be chemotactically active (Table III). Definite chemotactic activity was also found in the basic fragment obtained by short time treatment with plasmin.

*C. Inhibition of C3 derived anaphylatoxin by anti-C3 and anti-C3a:* Approximately 20 $\mu$g of low mol wt material with anaphylatoxin activity was added to 0.5 ml of anti-C3 and to 0.5 ml of a normal rabbit serum, respectively. After 30 min at 32°C, smooth muscle contracting activity was tested. As shown in

Fig. 13, anti-C3 completely abolished the anaphylatoxin effect, whereas normal rabbit serum did not noticeably interfere with the activity. Complete inhibition was also obtained in several experiments with an antiserum to C3a.

TABLE III

*Comparison of Chemotactic Activities Generated from C3 with Trypsin, C3 Convertase, and C3 Inactivator Complex*

| Sample | Treatment | Chemotactic value |
|---|---|---|
| *1 mg* | | |
| C3 | Trypsin, 10 μg, 20°C, 5 min* | 110‡ |
| — | Trypsin, 10 μg, 20°C, 5 min* | 32 |
| C3 | C3 convertase | 184‡ |
| — | C3 convertase | 98 |
| C3 | C3 inactivator complex | 351‡ |
| — | C3 inactivator complex | 88 |

* 20 μg soybean trypsin inhibitor added after treatment.

‡ Corrected for background activity of same amount of untreated C3.

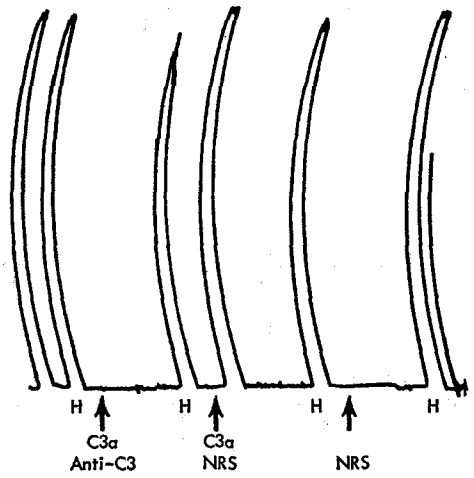


FIG. 13. Inhibition of anaphylatoxin activity by anti-C3 antiserum. 10 μg C3a were incubated with 0.2 ml of rabbit anti-human C3 or with 0.2 ml normal rabbit serum for 30 min at 32°C, after which the mixtures were applied to the guinea pig ileum. H indicates the effect of histamine.

*III. Description and Partial Purification of an Inactivator in Human Serum of C3- and C5-Derived Anaphylatoxin.*—

Addition of isolated C3a to samples of human serum resulted, within a few min, in total loss of its smooth muscle contracting activity. The inactivator of anaphylatoxin was shown to be a pseudoglobulin and to migrate, upon Pevikon

electrophoresis at pH 8.6, as an $\alpha$-globulin (Fig. 14). Upon sucrose density gradient ultracentrifugation, it sedimented with an $s$ rate of approximately 10S. When the $\alpha$-globulin fraction of the pseudoglobulins was submitted to further fractionation on a Sephadex G-200 column, the inactivator emerged slightly earlier than a 9.5S reference protein (Fig. 15). The active material from Sepha

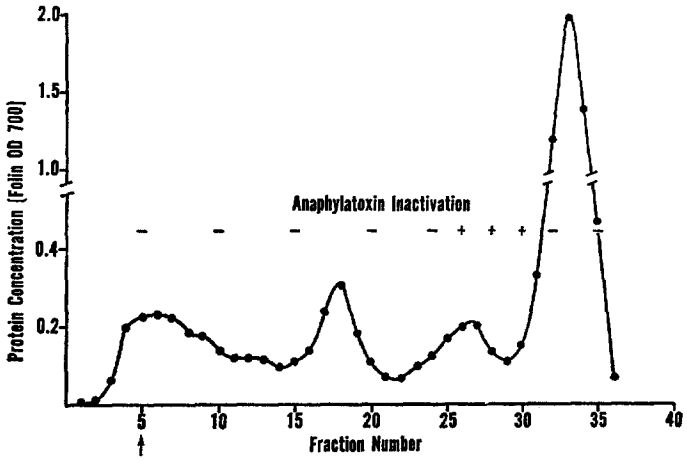


FIG. 14. Localization of anaphylatoxin inactivator in electrophoretic fraction of the pseudo globulins of human serum. Pevikon block; barbital buffer, pH 8.6, T/2 = 0.05; 24 hr; 3–4 v/cm. Arrow indicates origin; anode was at the right. Fractions 26–30 which contained the nactivator were pooled and concentrated for Sephadex filtration.

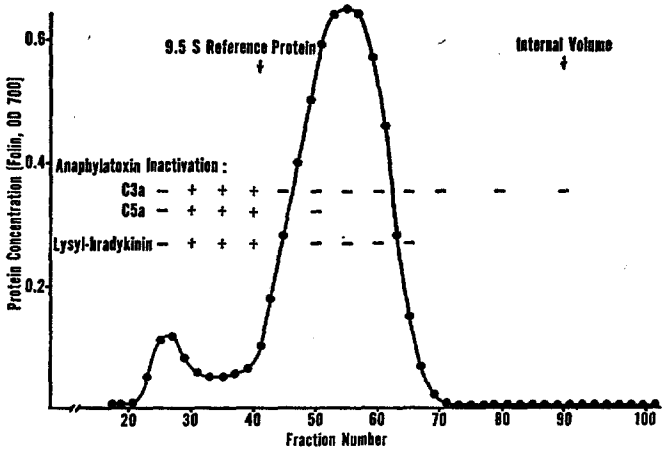


FIG. 15. Localization of anaphylatoxin inactivator in fractions obtained by Sephadex G-200 filtration of active material from Pevikon block (Fig. 14). The column was equilibrated with 0.15 M Tris buffer, pH 8.0. C3a, C5a and lysyl bradykinin were inactivated by the same fractions.

dex filtration was concentrated and subjected to disc electrophoresis at pH 8.7. Five protein bands could clearly be seen. Anaphylatoxin inactivator activity could be eluted from segments of unstained gels which corresponded to the position of one of the five protein bands. Further work is needed to isolate the inactivator.

The partially purified anaphylatoxin inactivator was found to be rapidly inactivated on heating at 56°C, but to be unaffected by soybean trypsin inhibitor or by incubation with $5 \times 10^{-3}$ M DFP. Its action on anaphylatoxin was dependent on time, temperature and pH; pH 5 and 0°C inhibited inactivation, whereas treatment for 5 min at room temperature at pH 7 or 8 led to inactivation of even a large molar excess of C3a (at least 100-fold). Evidence for binding of C3a by the inactivating factor could not be obtained. $^{125}$I-labeled C3a (prepared either by trypsin, $C\overline{4,2}$ or the C3 inactivator complex) exhibited the same ultracentrifugal behavior after inactivation by anaphylatoxin inactivator as untreated C3a controls. The radioactive material sedimented with an $s$ rate of 1–1.2S. The present evidence suggests an enzymatic mechanism of anaphylatoxin inactivation. The properties of the inactivator are summarized in Table IV.

TABLE IV

*Properties of Anaphylatoxin Inactivator*

| | |
|---|---|
| Solubility at $\mu = 0.02$, pH 5.4 | Soluble (pseudoglobulin) |
| Electrophoretic mobility at pH 8.6 | $\alpha$-globulin |
| Sedimentation velocity | Approximately 10S |
| Activity | Thermolabile (30 min, 56°C) |
| | pH dependent (pH 7–8) |
| | Temperature dependent |
| | Not inhibited by DFP or soybean trypsin inhibitor |

That the 10S $\alpha$-globulin described here has more general significance as a regulatory factor is indicated by two further observations. As illustrated in Fig. 15, column fractions containing the C3a inactivator also effected destruction of C5a anaphylatoxin and abolition of lysyl bradykinin activity.

## DISCUSSION

Recent work has shown that anaphylatoxin and chemotactic activity may be generated from C3 and C5. The development of this area of research and, in particular, the original evidence showing that these activities reside in low mol wt fragments of the respective complement components were recounted in a recently published review (18). Additional evidence in support of this concept was reported lately from several laboratories (3, 20, 21).

The present paper contributes to the available information the following

data and insights pertaining especially to formation, characteristics, isolation, and control of the biologically active C3 fragments. The percursor molecule, C3, possesses, in its native state, a highly susceptible region for attack by a variety of enzymes, including trypsin, thrombin, and plasmin. Enzymatic cleavage within this region leads to dissociation of a very basic, low mol wt fragment designated C3a. It was possible in this study to demonstrate C3a, for the first time directly as a protein entity, using acid polyacrylamide gel electrophoresis and to isolate the material in sufficient amounts for ultracentrifugal and electrophoretic analysis and for initiation of work on the structure of the protein, the results of which will be published elsewhere (15). Whereas anaphylatoxin activity has been derived from C3 heretofore only by means of C3 convertase or C3 inactivator complex, the present investigations showed that also trypsin and plasmin are able to liberate biologically active C3a and that thrombin sets free a very similar fragment which, however, was devoid of smooth muscle contracting activity.

Trypsin was particularly efficient in liberating C3a; the reaction approached completion within sec at room temperature even at relatively low enzyme concentrations. The piece removed by trypsin closely resembled C3a produced by C3 convertase in its behavior on disc electrophoresis, Sephadex filtration, and also with respect to its biological activity. It rendered the guinea pig ileum unresponsive to C3a (C3 convertase) but not to C5a anaphylatoxin (4). As will be reported elsewhere (15), the mol wts and amino acid compositions of C3a (trypsin) and C3a (C3 convertase) were exceedingly similar. Nevertheless, the two fragments differed in their C-terminal amino acid residues, indicating that the two enzymes cleave different bonds in the susceptible region of the C3 molecule. The close similarity between the two C3a fragments was also revealed by their yielding a reaction of identity with anti-C3 or anti-C3a in Ouchterlony plates. Furthermore, the anaphylatoxin activity of both could be inhibited with either of the two antisera.

Treatment of active C3a with trypsin rapidly abolished anaphylatoxin activity and caused the originally basic fragment to become negatively charged and to assume the electrophoretic mobility of a $\beta$-globulin. Since Ward (6) had previously demonstrated a chemotactically active anionic fragment of C3 after prolonged treatment of the protein with an equal amount of plasmin, the question was raised as to whether C3a might be chemotactic for polymorphonuclear leukocytes. It was clearly shown that the basic C3a fragment with anaphylatoxin activity also had chemotactic activity, irrespective of the type of enzyme used for its dissociation. However, unlike the smooth muscle contracting activity, the chemotactic activity persisted when C3a upon treatment with trypsin assumed a negative electrophoretic mobility. This finding indicated that both activities are a function of C3a, although of different chemical groups within the fragment, anaphylatoxin activity being apparently dependent upon

positively charged groups. The finding further suggested that the chemotactic C3 fragment described by Ward which was devoid of smooth muscle contracting activity is a degradation product of C3a. This view is supported by the observation that brief treatment of C 3 with plasmin gives rise to a basic fragment with anaphylatoxin as well as chemotactic activity.

In addition to formation and degradation of C3a, trypsin effects cleavage of the major fragment, C3b, into at least two antigenically distinct pieces, which were called C3c and C3d. This reaction proceeded with relative ease, being completed within 60 min at 37°C. The two pieces could readily be separated from each other utilizing their difference in size. Their availability will facilitate structural studies of C3, particularly in conjunction with studies of the chemical dissociation of the molecule by methods recently elaborated in this laboratory.[4] Material indistinguishable from the c- and d-fragments was obtained by treatment of C3b with thrombin or with plasmin. However, the efficiency of these enzymes in cleaving C3b was much lower than that of trypsin. There is no evidence to date that C3 convertase can cleave C3b; its high degree of substrate specificity apparently limits the enzyme to action on the susceptible region of native C3.

Aging of whole serum or brief incubation of fresh serum with antigen-antibody complexes was shown previously to result in conversion of C3 to C3i and in cleavage of C3i into $\beta_{1A}$- and $\alpha_{2D}$-globulin (22, 23). The present results indicate that C3i consists of a loose complex of C3a and C3b which may become completely dissociated at low pH. $\beta_{1A}$-globulin appears to be identical with C3c and $\alpha_{2D}$ with C3d. Considering also the secondary changes which C3a undergoes after its formation in serum, an antigen-antibody reaction in human serum may affect C3 in the following manner:

$$\mathrm{C3} \xrightarrow{\overline{\mathrm{C4,2}}} \mathrm{C3a,b} \qquad (1)$$

$$\mathrm{C3a} \xrightarrow{\text{Anaphylatoxin Inactivator}} \mathrm{(C3a)i} \qquad (2a)$$

$$\mathrm{C3b} \xrightarrow{\text{Serum enzyme}} \mathrm{C3c + C3d} \qquad (2b)$$

where C3a, b represents the associated form of the a- and b-fragment, which is prevalent at pH 7; where (C3a)i is the biologically inactive product of C3a and where "serum enzyme" is a noncomplement enzyme which may be thrombin.

The identity of the anaphylatoxin inactivator encountered in human serum is as yet uncertain. Circumstantial evidence for its existence had been presented by Dias Da Silva and Lepow, who were unable to generate anaphylatoxin activity with several fresh human sera tested (24). Lately, Lepow and

[4] Bokisch, V. A., and H. J. Müller-Eberhard. To be published.

Patrick (25) reported direct evidence for an inactivator in human serum and suggested that the inactivator acts by forming a complex with C3a rather than by acting on it enzymatically. The 10S $\alpha$-globulin described in this paper appears to act differently. It does not seem to neutralize C3a anaphylatoxin through the formation of an anaphylatoxin-inactivator complex. Rather, the conditions of the neutralization reaction suggest an enzymatic process. The inactivator is not strictly specific for C3a, but abolishes also the activities of C5a anaphylatoxin and of lysyl bradykinin. In view of the latter observation, the question arose as to whether the 10 S $\alpha$-globulin might be identical with serum kininase, i.e., carboxypeptidase N (26). Since information on the physicochemical properties of carboxypeptidase N is limited (27) and since a 10S inter-$\alpha$-globulin has not been described as a constituent of human serum (28), further work on this serum component is needed. Chemical studies are underway to define the effect of the inactivator on both anaphylatoxins and on lysyl bradykinin.

## SUMMARY

A small fragment of C3, called C3a, which has smooth muscle contracting activity, was isolated by three different methods. At pH 8.6, C3a behaved as cation, and using the Archibald method, its mol wt was determined to be 7000. A specific antiserum to C3a showed the fragment to be antigenically distinct from the rest of the C3 molecule, i.e., the C3b portion. The same antiserum and an anti-whole C3 were able to inhibit the biologic activity of C3a. In addition to anaphylatoxin activity, leukocyte chemotactic activity was shown to reside in C3a. Treatment with trypsin caused the cationic fragment to become anionic and abolished the anaphylatoxin but not the chemotactic activity.

C3a fragments with identical biologic activity and comparable cationic properties, as determined by acid disc electrophoresis, were obtained by treatment of C3 with C3 convertase, C3 inactivator complex, trypsin, and plasmin. Thrombin produced a similar C3 fragment which was inactive. It was concluded that C3a corresponds to an unusually basic portion of C3 which may be liberated by attack of a variety of enzymes on a highly susceptible region of the native C3 molecule.

C3b was cleaved by trypsin and less efficiently by thrombin or plasmin into two antigenically distinct pieces: the larger C3c fragment corresponding to $\beta_{1A}$ and the smaller C3d fragment to $\alpha_{2D}$ of aged serum. The c- and the d-fragments were separated and characterized.

Isolated C3a rapidly lost its anaphylatoxin activity when treated with small amounts of a partially purified, thermolabile 10S $\alpha$-pseudoglobulin of human serum. The conditions of inactivation suggested an enzymatic reaction. The anaphylatoxin inactivator also destroyed the activity of C5-derived anaphylatoxin and of lysyl bradykinin.

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