

## **Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase**

Viktor A. Bokisch, Hans J. Müller-Eberhard

*J Clin Invest.* 1970;49(12):2427-2436. <https://doi.org/10.1172/JCI106462>.

**Research Article**

The failure of human serum to give rise to anaphylatoxin activity could be attributed to the presence of a potent inactivator of anaphylatoxin in human serum. The inactivator was isolated and characterized as an  $\alpha$ -globulin with a molecular weight of approximately 310,000. It was found to abolish the activity of both anaphylatoxins, which are derived respectively from the third and the fifth component of complement, and of bradykinin. Inactivation of C3-derived anaphylatoxin and of bradykinin was accompanied by release of C-terminal arginine from these peptides. The anaphylatoxin inactivator was shown to hydrolyze the synthetic substrates hippuryl-L-arginine and hippuryl-L-lysine and to be inhibited by ethylenediaminetetraacetate (EDTA) or phenanthroline. These observations indicate that the anaphylatoxin inactivator constitutes a metal-dependent enzyme resembling in specificity pancreatic carboxypeptidase B.

**Find the latest version:**

<https://jci.me/106462/pdf>



# Anaphylatoxin Inactivator of Human Plasma: Its Isolation and Characterization as a Carboxypeptidase

VIKTOR A. BOKISCH and HANS J. MÜLLER-EBERHARD

*From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037*

**ABSTRACT** The failure of human serum to give rise to anaphylatoxin activity could be attributed to the presence of a potent inactivator of anaphylatoxin in human serum. The inactivator was isolated and characterized as an  $\alpha$ -globulin with a molecular weight of approximately 310,000. It was found to abolish the activity of both anaphylatoxins, which are derived respectively from the third and the fifth component of complement, and of bradykinin. Inactivation of C3-derived anaphylatoxin and of bradykinin was accompanied by release of C-terminal arginine from these peptides. The anaphylatoxin inactivator was shown to hydrolyze the synthetic substrates hippuryl-L-arginine and hippuryl-L-lysine and to be inhibited by ethylenediaminetetraacetate (EDTA) or phenanthroline. These observations indicate that the anaphylatoxin inactivator constitutes a metal-dependent enzyme resembling in specificity pancreatic carboxypeptidase B.

## INTRODUCTION

It has long been known that anaphylatoxin activity cannot be generated in human serum, whereas this activity is readily produced in a variety of animal sera, including rat, guinea pig, and hog serum. The question arose therefore as to whether human serum lacks the anaphylatoxin precursor or contains an inhibitor of anaphylatoxin. With the isolation and identification of complement proteins from human serum, it was possible in recent years to demonstrate that two distinct anaphylatoxins can be derived from the isolated third and fifth components of human complement (1, 2). Both anaphylatoxins are low

This is publication No. 418 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. 92037.

Dr. Bokisch is a Fellow of the Helen Hay Whitney Foundation.

Received for publication 27 May 1970.

molecular weight fragments, called C3a<sup>1</sup> and C5a, which arise during the complement reaction through the action of complement enzymes. Both peptides have the capacity to contract smooth muscle and to increase capillary permeability. These activities are primarily due to release of histamine from cellular elements and are therefore inhibitable by antihistamines.

Since anaphylatoxin could be produced from isolated human complement proteins but not from whole human serum, it was postulated that human serum contains an inactivator of anaphylatoxin (2). The presence of a heat-labile inactivator could subsequently be demonstrated in the  $\alpha$ -globulin fraction of human serum (3). The purpose of this communication is to report the isolation of the anaphylatoxin inactivator (AI), its enzymatic specificity, and its effect on both anaphylatoxins and on bradykinin. Emphasis will be placed on studies of the action of the inactivator on C3a anaphylatoxin, since this fragment to date has been more extensively characterized than C5a and has recently been found to exert strong biological activity in skin tests in humans (2).<sup>2</sup>

## METHODS

### Preparation of the anaphylatoxins from C3 and C5 of human complement

C3a anaphylatoxin was produced and isolated from highly purified C3 after treatment with the complement enzyme C3 convertase, trypsin, or the C3 inactivator complex according

<sup>1</sup> The symbols used have been recommended by the WHO Committee on Complement Nomenclature (1968). C2, C3, C4, and C5 denote, respectively, the second, third, fourth, and fifth components of the complement system. C4<sub>2</sub> designates the activating enzyme of C3, which is also called C3 convertase. EAC<sub>4,2,3</sub> refers to a complex consisting of erythrocytes, antibody and C4, C2, and C3. C3a and C5a refer to the biologically active, low molecular weight cleavage products of C3 and C5, respectively.

<sup>2</sup> Wuepper, K. D., V. A. Bokisch, H. J. Müller-Eberhard, and R. B. Stoughton. In preparation.

to methods described previously (3). C5a anaphylatoxin was produced from highly purified C5 by the action of trypsin or EAC4,2,3, as reported earlier (4).

### Isolation of the anaphylatoxin inactivator from human serum

**Preparation of pseudoglobulin.** Usually 500-ml batches of fresh human serum were processed in the following fashion. The conductance was lowered to 2.5 mmho/cm by dilution with approximately 2 liters of distilled water, and the pH was adjusted to pH 7.0 with 1 N HCl. After standing overnight at 4°C, the resulting precipitate was removed by centrifugation.

**Triethylaminoethyl (TEAE) cellulose chromatography.** The supernatant (approximately 2.5 liters) was applied to a 5 × 80 cm column containing approximately 1600 ml of packed TEAE cellulose which had been equilibrated with 0.02 M phosphate buffer, pH 7.0 (starting buffer I). Following the application of the sample, the column was washed with 4 liters of starting buffer. The protein was then eluted with a NaCl concentration gradient. A 3 liter beaker, which served as mixing chamber and contained 2500 ml of starting buffer, was connected by siphon to a second 3 liter beaker filled with 2500 ml of starting buffer containing 0.3 M NaCl. The flow rate was adjusted to 100 ml/hr and 20-ml fractions were collected. The fractions containing the AI were pooled and concentrated to approximately 50 ml in an Amicon concentration device using an XM 50 ultrafilter. The AI containing material from four separate TEAE cellulose columns was combined and then dialyzed against 0.02 M phosphate buffer, pH 7.0, containing 0.07 M NaCl (starting buffer II). It was then applied to a second TEAE column (3.5 × 50 cm) equilibrated with starting buffer II and the column was washed with 2 liters of this buffer before a NaCl concentration gradient was started. A 2 liter beaker containing 1500 ml of starting buffer II served as mixing chamber and was connected by siphon with another 2 liter beaker filled with 1500 ml 0.02 M phosphate buffer, pH 7.0, containing 0.25 M sodium chloride. The flow rate was adjusted to approximately 70 ml/hr and fractions of 20 ml were collected. The fractions containing the AI were pooled and concentrated to 10 ml as described above.

**Pevikon block electrophoresis.** The material was dialyzed overnight against barbital buffer, pH 8.6, ionic strength 0.05, and subsequently subjected to Pevikon block electrophoresis in the same buffer. Electrophoresis was carried out for 20 hr using a potential gradient of 4 v/cm. Fractions containing the AI were concentrated to 10 ml and again subjected to electrophoresis using the same conditions. The fractions containing the activity were pooled and concentrated to 5 ml.

**Gel filtration on Sephadex G-200.** Sephadex filtration was carried out using a 3.5 × 170 cm Sephadex G-200 column equilibrated with 0.05 M phosphate buffer, pH 7.0. The sample was layered between the top of the column and the supernatant buffer. The flow rate was adjusted to 15 ml/hr and fractions of 3.5 ml were collected. Fractions containing the AI were pooled, concentrated to 2 ml, and applied to a second G-200 column (2.3 × 100 cm) equilibrated with the same buffer. The flow rate was adjusted to 10 ml/hr and fractions of 3 ml were collected. Fractions containing the AI were pooled and concentrated to a protein concentration of approximately 50 µg/ml, distributed into 0.5 ml aliquots, frozen in liquid nitrogen, and stored at -70°C.

### Rabbit antisera

Anti-AI was produced by injecting 20 µg of AI in complete Freund's adjuvant into the popliteal lymph nodes of rabbits. 4 wk later these animals were boosted three times at weekly intervals by injecting the same dose intramuscularly. The antisera obtained reacted strongly with AI forming a precipitin band on Ouchterlony and immunoelectrophoretic analysis. In order to test whether the antibody was capable of inhibiting AI activity, a dose of AI sufficient to inactivate 5 µg of C3a was incubated for 30 min at 37°C with either 0.3 ml of antiserum or the equivalent amount of γ-globulin isolated from this antiserum by Pevikon block electrophoresis. The antisera were heated for 30 min at 56°C before use in order to abolish anaphylatoxin inactivator activity present in rabbit serum.

Antisera to α<sub>2</sub>-macroglobulin, α<sub>2</sub>-lipoprotein, and haptoglobin were purchased from Behringwerke AG, Marburg/Lahn, Germany.

### Molecular weight determinations

The molecular weight of the AI was calculated from the sedimentation (s) and diffusion coefficient (D), assuming a partial specific volume of 0.73. s was estimated by ultracentrifugation in a 6.9–31.0% linear sucrose density gradient using an L2 Spinco machine and an SW 50 rotor. D was estimated according to Andrews (5) employing a 3.5 × 170 cm Sephadex column equilibrated with 0.05 M phosphate buffer, pH 7.0. Reference substances used were: equine cytochrome c ( $13 \times 10^{-7}$  cm<sup>2</sup>/sec), hemoglobin ( $6.8 \times 10^{-7}$  cm<sup>2</sup>/sec), transferrin ( $5.0 \times 10^{-7}$  cm<sup>2</sup>/sec), γG globulin-<sup>125</sup>I ( $3.8 \times 10^{-7}$  cm<sup>2</sup>/sec), and thyroglobulin-<sup>125</sup>I ( $2.5 \times 10^{-7}$  cm<sup>2</sup>/sec).

The molecular weight was also determined employing the polyacrylamide gel electrophoresis method according to Hedrick and Smith (6). The gel concentrations ranged from 6 to 10%. As reference substances with known molecular weights were used: albumin in its monomeric, dimeric, and trimeric form (mol wt 65,000, 130,000, and 195,000, respectively) and urease (mol wt 485,000), Code: URC, Worthington Biochemical Corp., Freehold, N. J. Bromphenol blue served as reference for determination of the R<sub>m</sub> of the various proteins. Its position at the end of electrophoresis was marked by the tip of a copper wire inserted prior to staining of the gels. The logarithm of the relative distance of migration of the proteins was plotted against gel concentration and the slope of this plot was calculated and graphically related to the molecular weight of the reference proteins.

### Analytical polyacrylamide gel electrophoresis

This was carried out according to Davis (7) using a gel concentration of 6% and Tris-HCl-glycine buffer, pH 8.7.

### Cellulose acetate strip electrophoresis

This was performed in a Beckman microzone electrophoresis apparatus, Model R101, using Beckman barbital buffer B-2, pH 8.5, ionic strength 0.075 and 250 v for 20 min at 20°C.

### Assay of biological activity

Anaphylatoxin activity was assayed on segments of isolated guinea pig ileum as described previously (4). 4–5 µg of C3a was used for each individual determination. A similar

amount of C5a was used which was produced by treatment of 150  $\mu\text{g}$  of C5 with trypsin or EAC<sub>4,2,3</sub>. C5a was employed without prior separation from the reaction mixture in which it was produced. AI activity was assayed by verifying abolition of the activity of C3a or C5a after incubation of these peptides with aliquots of AI for 2 min at 20°C and pH 7.5.

C3a activity was also tested on estrous rat uterus. Virgin rats, weighing 150 g, were injected intramuscularly with 0.1 ml of a diethylstilbestrol solution. This solution was prepared by diluting 0.1 ml of 5 mg/ml diethylstilbestrol (Eli Lilly and Company, Indianapolis, Ind.) with 9.9 ml of cottonseed oil; approximately 20 hr after injection the rats were sacrificed and the uterus was isolated. One horn of the uterus was placed in a 20 ml bath containing Tyrode solution. By pretreatment of the muscle with larger doses of serotonin (serotonin, creatinine sulfate, Batch 511786, Sandoz Pharmaceuticals, Hanover, N. J.) and bradykinin (bradykinin triacetate, Lot H2351, Cyclo Chemical Division Travenol Laboratories, Inc., Los Angeles, Calif.) the uterus was rendered responsive to 0.2  $\mu\text{g}$  of serotonin and 5–10 ng of bradykinin. None of the 10 muscles used responded to histamine. C3a was injected into the bath in amounts of 3–4  $\mu\text{g}$ . To test for serotonin release by C3a, 2  $\mu\text{g}$  of antiserotonin (methysergide maleate, research material, Sandoz Pharmaceuticals) was added to the bath 2 min before application of C3a.

Bradykinin activity was assayed on estrous rat uterus. 5–10 ng of untreated or AI-treated bradykinin were applied to the bath. To demonstrate inactivation, 380  $\mu\text{g}$  of bradykinin was incubated with 24  $\mu\text{g}$  AI for 8 hr at 37°C and pH 7.5.

### High voltage electrophoresis

The effect of the AI on C3a and bradykinin was analyzed on high voltage electrophoresis using Whatman chromatography paper 3 MM and a pyridine acetic acid buffer of pH 6.4. The buffer consisted of 24 ml of glacial acetic acid, 600 ml of pyridine, and 5400 ml of distilled water. Electrophoresis was carried out in a high voltage electrophorator, Model D, Gilson Medical Electronics, Inc., Middleton, Wis., for 90 min applying 1250 v. For analytical purposes 15 nmoles of C3a or bradykinin was applied. After drying, the paper was stained with a solution of 0.5% ninhydrin in acetone. Arginine was used as marker. Insulin and ribonuclease were also tested as possible substrates of AI. After treatment with AI, 15 nmoles of insulin (Eli Lilly and Co.) or ribonuclease (crystalline 5X, Nutritional Biochemical Corp., Cleveland, Ohio) was applied to the paper.

For preparative purposes, 150 nmoles of C3a or bradykinin after treatment with AI was streaked out on the paper, applying 100 nmoles/cm. After electrophoresis a small strip was cut out of the paper and was stained with ninhydrin. This strip was lined up with the paper and the area corresponding to the ninhydrin stain was cut out of the unstained paper and eluted with 5 ml of 0.1 M ammonium bicarbonate buffer, pH 7.9, for 20 hr. The eluate was subsequently subjected to further analysis.

### Peptide mapping of C3a

The peptide map of the trypsin digest of C3a was obtained by combining paper chromatography with high voltage electrophoresis at pH 3.6 according to Katz, Dreyer, and Anfinsen (8). C3a was oxidized with performic acid (9); lyophilized material was dissolved in 0.6 ml of a solution prepared with 5 parts of 97% performic acid and 1 part

methanol. 1 ml of performic acid reagent was then added and the mixture was kept at  $-5^{\circ}\text{C}$  for 150 min. The performic acid reagent was prepared by mixing 95 parts of 97% performic acid with 5 parts of hydrogen peroxide. This mixture was kept for 120 min at 20°C before use. After oxidation of C3a the mixture was diluted with 40 ml of distilled water and lyophilized. The material was subsequently dissolved in 2 ml 0.1 M ammonium bicarbonate buffer, pH 7.9, and treated with 1% trypsin (w/w) at 20°C for 2 hr. The digest was then lyophilized and redissolved in 50  $\mu\text{l}$  of water and applied to the paper. For analytical purposes, 15 nmoles, and for preparative purposes, 150 nmoles of C3a were applied to the paper. Descending chromatography was performed for 20 hr at 20°C using as solvent a mixture containing 720 ml of butyl alcohol, 213 ml of glacial acetic acid, and 1066 ml of water. Following chromatography the paper was subjected to electrophoresis, applying 2500 v for 70 min. The buffer in which the electrophoresis was carried out was prepared by mixing 25 ml of pyridine, 250 ml of glacial acetic acid, and 7225 ml of distilled water. For analytical purposes the paper was stained with a solution of 0.5% ninhydrin in acetone. If elution of peptides was intended, the paper was stained with 0.02% ninhydrin solution. Areas showing ninhydrin stain were cut out and eluted as described above.

### Analysis of the effect of AI and carboxypeptidase B on the C-terminal residue of C3a

50 nmoles of C3a in 2 ml of 0.1 M ammonium bicarbonate buffer, pH 7.9, were incubated with either 0.2 nmoles of AI or 2.5 nmoles of carboxypeptidase B (CPB) (Code: COBDFP, Worthington Biochemical Corp.) for 30 min at 37°C. In order to investigate a possible effect of carboxypeptidase A (CPA) on C3a, 50 nmoles of C3a was treated with 2.5 nmoles of CPA (Code: COADFP, Worthington Biochemical Corp.) under the conditions described above. The reaction was stopped by increasing the hydrogen ion concentration to pH 2.5–3.0. 50 nmoles of norleucine (DL-norleucine, Lot 100177, California Foundation for Biochemical Research, Los Angeles, Calif.) were incorporated into the mixture as internal standard. The reaction mixture was then passed over a 3 cm column containing 2 ml of Dowex 50 H<sup>+</sup> (AG 50 W X 8, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 5 ml of distilled water and subsequently the adsorbed amino acids were eluted with 3 ml of 3 N ammonium hydroxide. After evaporation the samples were subjected to quantitative amino acid analysis using a Beckman Spinco amino acid analyzer.

### Effect of AI and CPB on synthetic substrates

1 ml of a  $7 \times 10^{-4}$  M solution of hippuryl-L-arginine or hippuryl-L-lysine (Mann Research Laboratories, New York) in 0.025 M Tris buffer, pH 7.6, containing 0.1 M NaCl were filled into quartz cuvettes having a 1 cm light path. 0.1 ml of the same buffer containing either 0.14  $\mu\text{g}$  CPB, 1.4  $\mu\text{g}$  CPB, 1.4  $\mu\text{g}$  AI, or 14  $\mu\text{g}$  AI was pipetted into the substrate solutions at zero time and the absorbency at 254  $m\mu$  was determined spectrophotometrically at various time intervals. The reaction was carried out at 25°C.

### N-terminal amino acid determination

N-terminal analysis of bradykinin and bradykinin after inactivation with the AI was carried out according to Gray and Hartley (10).

TABLE I  
Effect of Various Sera on Anaphylatoxin Activity

Serum	Type of anaphylatoxin	Inactivation*	Frequency
Human	Human C3a	+	30/30
Human	Human C5a	+	5/5
Human	Guinea pig	-	2/2
Human	Rat	-	4/4
Guinea pig	Human C3a	+	3/3
Guinea pig	Human C5a	+	2/2
Guinea pig	Guinea pig	-	4/4
Guinea pig	Rat	-	2/2
Rat	Human C3a	+	5/5
Rat	Human C5a	+	3/3
Rat	Guinea pig	-	2/2
Rat	Rat	-	5/5
Rabbit	Human C3a	+	2/2
Rabbit	Human C5a	+	3/3
Rabbit	Guinea pig	-	2/2
Rabbit	Rat	-	2/2

\* 4  $\mu$ g of C3a anaphylatoxin treated for 2 min at 20°C with 20  $\mu$ l of undiluted serum. The average volume of anaphylatoxin containing guinea pig and rat serum was respectively 0.4 ml and 0.05 ml. Anaphylatoxin activity was generated by incubation of guinea pig or rat serum with 50  $\mu$ g/ml of isolated cobra venom factor for 20 min at 37°C.

## RESULTS

*Inactivation of human C3a- and C5a-anaphylatoxin by various sera.* As summarized in Table I, human C3a and C5a were inactivated within 2 min at 20°C by all sera tested, including samples of human, guinea pig, rat, and rabbit serum. Usually 15  $\mu$ l of undiluted serum was sufficient to cause inactivation. Plasma prepared from freshly drawn, heparinized human blood was equally active as serum. In contrast, anaphylatoxin generated in whole guinea pig and rat serum was neither inactivated by the autologous serum nor any of the heterologous sera tested. The data suggest that the inactivating principle in human serum also occurs in various animal sera, although in guinea pig and rat serum it does not appear to affect the autologous anaphylatoxins.

*Isolation of the anaphylatoxin inactivator from human serum.* Since AI has previously been found to be a pseudoglobulin, serum from which the low ionic strength precipitate was removed was used as starting material. Three qualitatively different separation steps were employed consecutively for the isolation of AI (Fig. 1). First, 500 ml of starting material was chromatographed on TEAE-cellulose in phosphate buffer, pH 7.0 utilizing a NaCl concentration gradient. AI activity was eluted at a conductivity range of 10–12 mmho/cm. Following

concentration and pooling of the active material from four separate experiments, TEAE chromatography was repeated using slightly different gradient elution conditions. Fractions containing AI activity from the second chromatogram were pooled and concentrated to 10 ml and subjected to electrophoresis on Pevikon in barbital buffer, pH 8.6. The protein of the  $\alpha$ -globulin region where AI activity was invariably detected was concentrated and again subjected to electrophoresis under identical conditions. The active fraction, concentrated to 5 ml, was then passed over a column of Sephadex G-200, where the activity emerged near the exclusion volume. Also, this procedure was repeated with the active material utilizing a smaller Sephadex column. As shown in Fig. 1f, the distribution of AI activity upon gel filtration in the final isolation step corresponded closely to the distribution of protein.

The average yield of AI was 2 mg/liter of serum. Approximately 0.5  $\mu$ g of AI was equivalent to 15  $\mu$ l of serum with respect to anaphylatoxin inactivation. This amount corresponds to 1/4000 of the average yield and to the activity contained in 60 ml of human serum. Assuming that no inactivation occurred during the isolation procedure, the over-all yield of AI was 6%. On the basis of these figures the amount of AI in serum may be estimated to be 30–40  $\mu$ g/ml. Preliminary immunochemical quantitation gave a comparable value for AI concentration in human serum.

*Molecular properties of isolated anaphylatoxin inactivator.* The physicochemical and immunochemical homogeneity of isolated AI is demonstrated in Fig. 2 which shows the results of disc electrophoretic and immunoelectrophoretic analyses. AI behaves on electrophoresis in agar or in Pevikon as an  $\alpha$ -globulin. The sedimentation coefficient was found to be approximately 9.5S and the diffusion coefficient to be  $2.9 \times 10^{-7}$  cm<sup>2</sup>/sec. The molecular weight was calculated from  $s$  and  $D$  assuming a  $\bar{V}$  of 0.73 to be 325,000. By the polyacrylamide gel electrophoretic method a molecular weight of approximately 290,000 was obtained (Fig. 3). These data are summarized in Table II. Isolated AI gave negative reactions with antisera to human  $\alpha_2$ -macroglobulin,  $\alpha_1$ -lipoprotein, and haptoglobin.

*Activity of isolated anaphylatoxin inactivator.* In 30 separate experiments, 0.4–0.5  $\mu$ g of AI was found to cause inactivation 4–5  $\mu$ g of C3a anaphylatoxin in 2 min at 20°C. In these experiments C3a was produced either by treatment of C3 with trypsin, or C4<sub>2</sub> or C3 inactivator complex, and all preparations were equally affected by AI. C5a anaphylatoxin produced either by treatment of C5 with trypsin or with EAC4<sub>2,3</sub> was also inactivated by AI, as observed in five different experiments, and the quantitative conditions required for the effect were identical with those described for C3a.

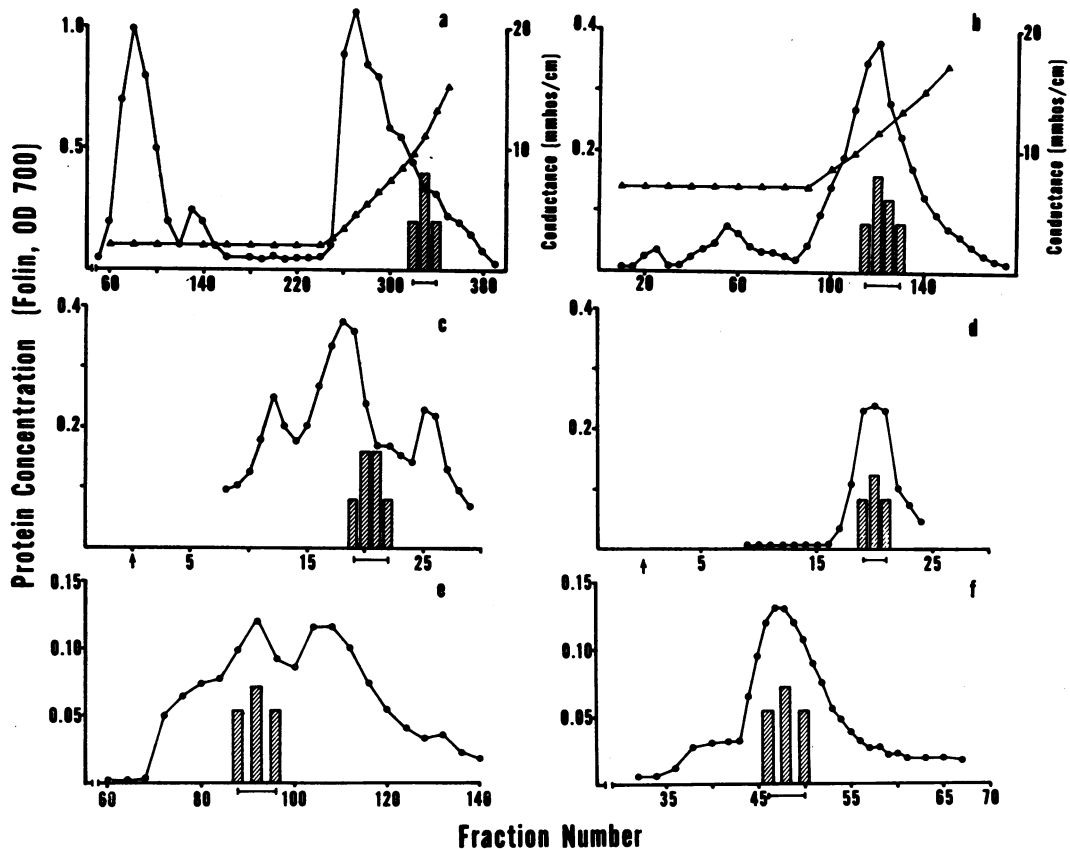


FIGURE 1 Isolation of AI from human serum. Three different preparative steps were employed and each step was repeated once. *a,b*: chromatography on TEAE-cellulose at pH 7.0; *c,d*: Pevikon block electrophoresis at pH 8.6; *e,f*: gel filtration on Sephadex G-200. Pseudoglobulin was used as starting material. Solid circles indicate the distribution of protein and the shaded columns show the relative AI activity. Fractions containing AI activity were pooled as indicated by the bars. The triangles in *a* and *b* indicate the conductance of the elution buffer. The arrows in *c* and *d* designate the origin.

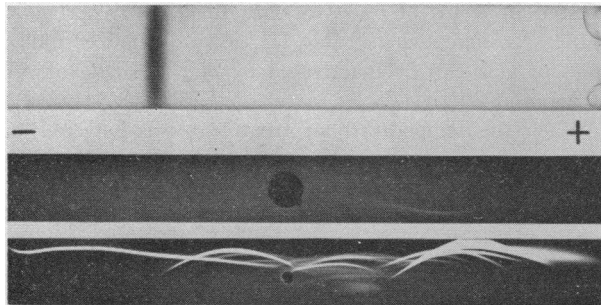


FIGURE 2 Demonstration of AI on disc gel electrophoresis (upper panel) and on immunoelectrophoresis (lower panel). The immunoelectrophoretic analysis is a composite of two different experiments; the upper part shows AI which was developed with a rabbit anti-human AI, and the lower part shows for comparison the pattern of human serum.

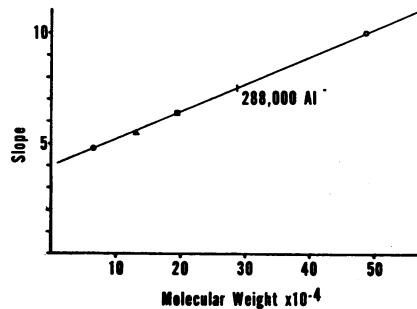


FIGURE 3 Molecular weight determination of AI by disc gel electrophoresis. The slopes were obtained by plotting the  $R_m$  values of each substance vs. gel concentration. As reference substances served albumin monomer ( $\bullet$ ), dimer ( $\blacktriangle$ ), trimer ( $\blacksquare$ ), mol wt 65,000, 130,000, and 195,000, respectively, and urease ( $\circ$ ), mol wt 485,000.

TABLE II  
Properties of the Anaphylatoxin Inactivator

Solubility at $\mu = 0.02$ , pH 5.4:	Soluble (pseudoglobulin)
Electrophoretic mobility, pH 8.6:	$\alpha$ -Globulin
Sedimentation velocity:	9.5
Diffusion coefficient:	2.9
Mol wt:	325,000
Activity:	Thermolabile; abolished below pH 5 and above pH 10; inhibited by $10^{-2}$ M EDTA, $10^{-2}$ M phenanthroline
Action	Temperature and pH dependent 1 molecule of AI inactivates 300-400 molecules of C3a in 2 min at 20°C

The biological activity of C3a and C5a was assayed by measuring the contraction of an isolated segment of the guinea pig ileum in a 20 ml bath. Fig. 4 shows the recording of a representative experiment performed with a single ileum segment. To preclude misinterpretation of the AI effect as tachyphylaxis, AI-treated C3a was applied first, and following a wash, untreated C3a was injected into the bath. Whereas treated C3a invariably failed to elicit a contraction, a full contraction was observed with untreated C3a. A subsequent application of an identical dose of C3a was without effect, indicating that now the muscle was tachyphylactic to C3a. As anticipated, the muscle retained its reactivity toward C5a anaphylatoxin, so that the same segment could be used to show that the effect of AI on C5a was identical with its effect on C3a.

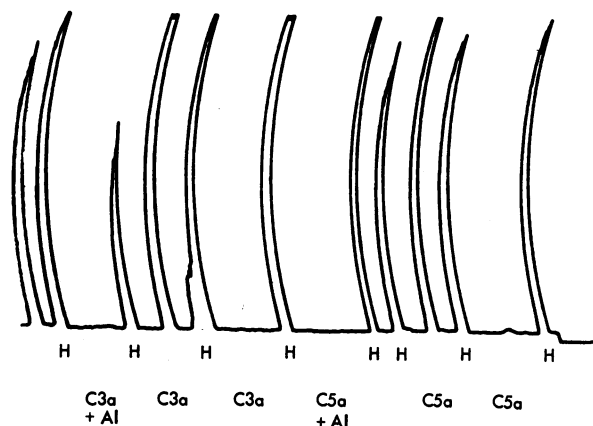


FIGURE 4 Inactivation of C3a and C5a anaphylatoxin by treatment with AI. 4-5  $\mu$ g of C3a or C5a were incubated with aliquots of AI for 2 min at 20°C and pH 7.5, and subsequently applied to the guinea pig ileum (C3a + AI, C5a + AI). Following treatment with AI, C3a or C5a had no effect on the ileum, whereas identical amounts of untreated C3a or C5a caused contraction. The failure of the ileum to respond to a second application of C3a or C5a is due to desensitization. Since C3a and C5a do not cross-desensitize the ileum, the same muscle segment could be used for these experiments. H indicates the effect of histamine.

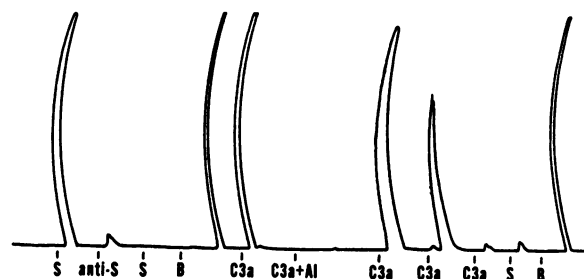


FIGURE 5 Contraction of the rat uterus by C3a anaphylatoxin and abolition of this activity by treatment with AI. Application of 3-4  $\mu$ g of C3a caused contraction of the rat uterus. Treatment of C3a with aliquots of AI for 2 min at 20°C and pH 7.5 abolished this activity (C3a + AI). Repeated applications of C3a rendered the uterus gradually unresponsive. Treatment of the uterus with anti-serotonin (methysergide maleate) did not affect its susceptibility to C3a. S, anti-S, and B indicate administration of 0.2  $\mu$ g serotonin, 2  $\mu$ g of anti-serotonin, and 8 ng of bradykinin, respectively.

observed with untreated C3a. A subsequent application of an identical dose of C3a was without effect, indicating that now the muscle was tachyphylactic to C3a. As anticipated, the muscle retained its reactivity toward C5a anaphylatoxin, so that the same segment could be used to show that the effect of AI on C5a was identical with its effect on C3a.

C3a activity was also assayed by measuring the contraction of the estrous rat uterus in a 20 ml bath (Fig. 5). This activity was not inhibitable by antihistamine (chlorpheniramine maleate) or by antiserotonin (methysergide maleate) and resembled in this respect kinin activity. However, unlike kinins, C3a was capable of inducing tachyphylaxis of the uterus. The activity was readily abolished by treatment of C3a with AI. The amounts used of C3a and AI were comparable to those employed in the guinea pig ileum assay.

Various preparations of AI were analyzed for anaphylatoxin inactivating activity and their potency was expressed in terms of moles of C3a inactivated per mole of AI. As indicated in Table III, several hundred molecules of C3a can be inactivated by one molecule of AI in 2 min at 20°C.

When the temperature of treatment was lowered to +1°C, no inactivation of C3a by AI was detected during 5 min. When treatment of C3a was carried out at 20°C no effect of AI was noted at pH 5 and below. Inhibition of the reaction at low pH was not due to destruction of AI activity, since adjustment of the hydrogen ion concentration to pH 7 after 5 min of exposure of pH < 5 resulted in full restoration of AI activity. However, exposure of AI to pH 4 for 1 hr at 20°C destroyed its activity irreversibly. These observations indicated a pH and temperature dependence of the action of AI on C3a.

TABLE III  
Quantitation of the Anaphylatoxin Inactivator Effect on C3a and Abolition of the AI Activity by Various Treatments

AI preparation	Molecules C3a/molecule AI	Condition of inactivation of C3a
No. 1 untreated*	500	0.5 min, 20°C
No. 2 untreated	100	4 min, 37°C
No. 3 untreated	225	5 min, 37°C
No. 4 untreated	530	2 min, 20°C
No. 5 untreated	810	2 min, 20°C
No. 6 (Veronal buffer, 40 hr, 4°C)	500	2 min, 20°C
No. 6 (0.01 M EDTA, 40 hr, 4°C)	12	2 min, 20°C
No. 6 (0.01 M phenanthroline, 40 hr, 4°C)	24	2 min, 20°C
No. 4 (300 µl normal rabbit serum, 30 min, 37°C)	530	2 min, 20°C
No. 4 (300 µl rabbit anti-AI, 30 min, 37°C)	200	2 min, 20°C

\* The absolute amount of AI used in these experiments ranged from 0.4 to 1.5 µg.

*Chemical nature of AI effect on C3a.* Cellulose acetate electrophoresis revealed that loss of anaphylatoxin activity of C3a through treatment with AI was accompanied by a change in electrophoretic mobility. The inactivated C3a migrated less rapidly to the cathode than active C3a (Fig. 6).

The apparent loss of positive net charge could be explained by the liberation of arginine resulting from the action of AI. Free arginine was first demonstrated in the reaction mixtures of C3a and AI by high voltage electrophoresis. Quantitative amino acid analysis performed after removal of the protein from the mixture indicated the presence of approximately 1 mole of arginine per mole of treated C3a (Table IV). No other amino acids could be detected. Since arginine was previously found to be in C-terminal position of C3a anaphylatoxin, and serine in N-terminal position,<sup>8</sup> the liberated arginine could only represent the C-terminal residue. Carboxypeptidase B (CPB), which previously was shown to cleave arginine from C3a,<sup>8</sup> was therefore tested for its effect on the activity of C3a. Treatment of C3a for 2 min at 20°C with CPB in a molar ratio of 50:1 completely abolished anaphylatoxin activity. In contrast, carboxypeptidase A (CPA) in equimolar ratio had no effect on C3a activity.

<sup>8</sup> Budzko, D. B., V. A. Bokisch, and H. J. Müller-Eberhard. Anaphylatoxin of the third component of human complement: chemical studies of the precursor and its biologically active fragments produced by C3 convertase, trypsin or hydroxylamine. Submitted for publication.

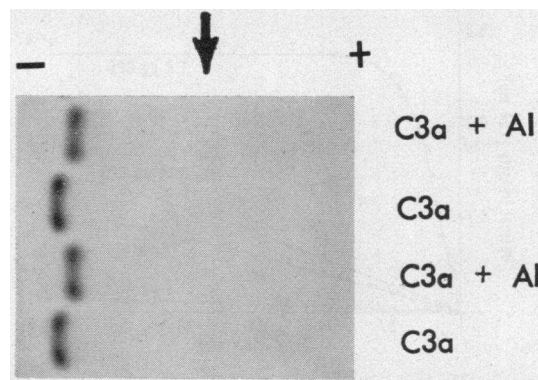


FIGURE 6 Effect of AI on the electrophoretic behavior of C3a on cellulose acetate at pH 8.5. Following treatment a shift of the protein band into a more anodal position is discernible. The arrow indicates the line of application.

The encountered resemblance between AI and CPB activity prompted the following experiments. Extensive dialysis of AI against 0.01 M ethylenediaminetetraacetate (EDTA) or phenanthroline markedly reduced AI activity (Table III), suggesting the presence in the enzyme of a functionally essential bivalent metal ion. Comparison of the effect of AI and CPB on hippuryl-L-arginine or hippuryl-L-lysine showed that AI definitely hydrolyzed these synthetic substrates. The rate of hydrolysis of hippuryl-L-arginine, however, was only approximately 1/15th of that observed with CPB (Fig. 7). AI failed to liberate the C-terminal residues from ribonuclease and insulin. It may be concluded from these results that human serum AI has an enzymatic specificity resembling that of pancreatic CPB.

Fig. 8 compares the tryptic peptide maps of untreated C3a and C3a treated with AI. A single peptide was clearly affected which was the most positively charged peptide present, as evidenced by its extreme cathodal location. Amino acid analysis of the eluted and hydrolyzed peptide revealed the presence of arginine in addition to several other amino acids. The AI-induced shift of this peptide toward the anode is consistent with removal of an arginine residue.

*Inactivation of bradykinin by AI.* To test whether AI effected inactivation of kinins, 380 µg of bradykinin was treated with 24 µg of AI for 8 hr at 37°C, and small

TABLE IV  
Liberation of Arginine from C3a by Anaphylatoxin Inactivator and by Carboxypeptidase B

Enzyme	Molar ratio Substrate/Enzyme	Conditions	Molecule residue per molecule C3a
AI	3000/1	30 min, 37°C	0.68 Arg
CPB	20/1	30 min, 37°C	0.7 Arg



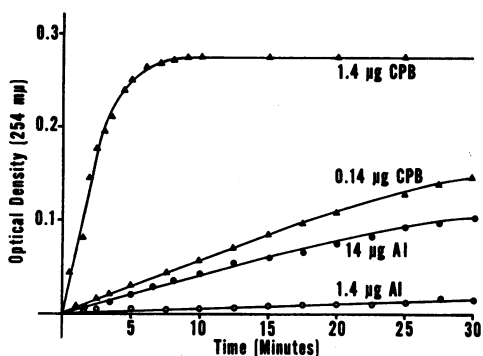


FIGURE 7 Hydrolysis of hippuryl-L-arginine by AI and CPB. Aliquots of a solution of  $7 \times 10^{-4}$  moles hippuryl-L-arginine were treated with  $1.4 \mu\text{g}$  AI,  $14 \mu\text{g}$  AI,  $0.14 \mu\text{g}$  CPB, and  $1.4 \mu\text{g}$  CPB for 30 min at  $25^\circ\text{C}$  and pH 7.6. Hydrolysis of the substrate was measured spectrophotometrically. Compared at equimolar concentrations, CPB was approximately 15 times more efficient than AI.

aliquots of the reaction mixture and of the control which lacked AI were examined for kinin activity. As shown in Fig. 9, bradykinin was inactivated by AI under these conditions.

High voltage electrophoresis of AI-treated bradykinin from the same reaction mixture showed release of arginine (Fig. 10). Since bradykinin has arginine in N- and in C-terminal position, the inactivated bradykinin was eluted from the paper and subjected to N-terminal residue analysis. By dansylation, N-terminal arginine was demonstrated to be present, indicating that AI inactivated bradykinin by liberating the C-terminal arginine.

## DISCUSSION

The protein described appears to be a heretofore unrecognized serum constituent. It is an  $\alpha_1$ -globulin with a

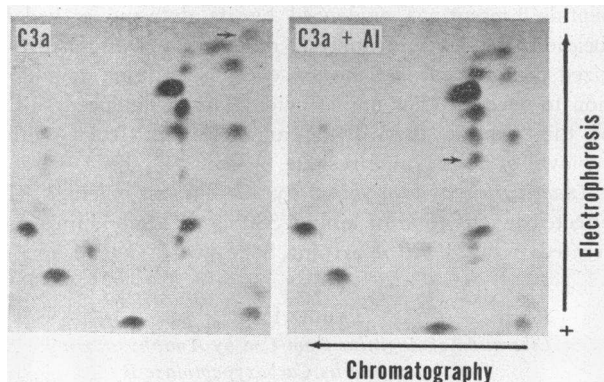


FIGURE 8 Tryptic peptide maps of C3a. The left panel shows the map of untreated C3a and the right panel that of C3a treated with AI. The point of application of the sample was in the lower right corner of the paper. Electrophoresis was carried out at pH 3.5. AI affected only the most positively charged peptide, which changed its position as indicated by the arrows.

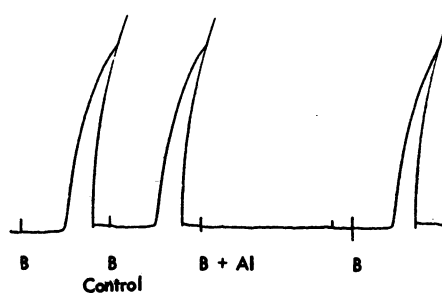


FIGURE 9 Inactivation of bradykinin by treatment with AI.  $380 \mu\text{g}$  of bradykinin was incubated with  $24 \mu\text{g}$  of AI for 8 hr at  $37^\circ\text{C}$  and pH 7.5. Aliquots of the reaction mixture containing 5 ng of bradykinin were applied to the uterus (B + AI). B control: bradykinin incubated at  $37^\circ\text{C}$  for 8 hr without AI; B: untreated bradykinin.

sedimentation rate of 9.5S and a molecular weight of approximately 310,000 and it occurs in human serum in a concentration of 30–40  $\mu\text{g}/\text{ml}$ . An  $\alpha_1$ -globulin with a similarly high molecular weight has not been recorded in the literature to our knowledge, except  $\alpha_1$ -lipoprotein, which, however, has a sedimentation rate of 5.5S and a serum concentration 100 times that of AI (11). Further, comparative immunochemical analysis of the two proteins failed to reveal an antigenic relationship. The average yield of 6% for isolated AI compares favorably with that of other trace proteins of human serum, for instance, the second component of complement (12).

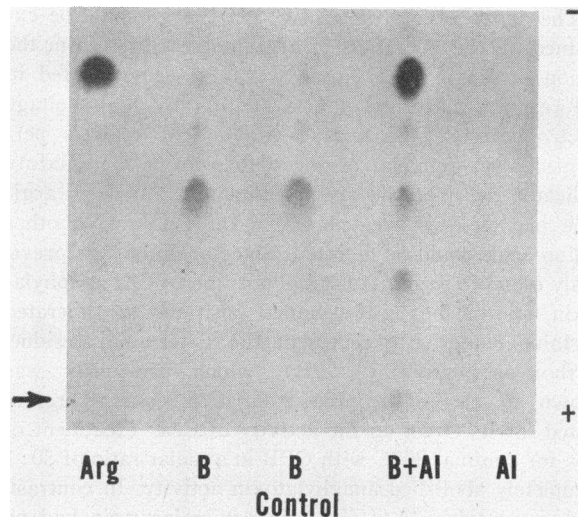


FIGURE 10 Release of arginine from bradykinin by treatment with AI demonstrated by high voltage electrophoresis at pH 6.4.  $380 \mu\text{g}$  of bradykinin was treated with  $24 \mu\text{g}$  AI for 8 hr at  $37^\circ\text{C}$  and pH 7.5 (B + AI). B control: bradykinin incubated for 8 hr at  $37^\circ\text{C}$  without AI; B: untreated bradykinin; Arg: arginine. The arrow points at the line of application of the samples.

That AI is an enzyme was suggested by the pH and temperature dependence of its action and the fact that one molecule of AI can effect inactivation of many hundred molecules of C3a. Its enzyme nature is clearly revealed by the finding that AI removes the C-terminal residue from both C3a and bradykinin. Since arginine is in C-terminal position in both peptides, it may be concluded that AI is endowed with carboxypeptidase B specificity. Indeed, it could be shown that AI was capable of hydrolyzing typical CPB substrates, whereas it failed to liberate the C-terminal residues from ribonuclease and insulin which are attacked by CPA. The proposed relationship to CPB is supported by experiments showing that pancreatic CPB in removing the C-terminal arginine from C3a also abolished its activity. Further, like CPB, AI was inactivated by treatment with phenanthroline or EDTA. Although it resembles pancreatic CPB with respect to enzyme specificity, AI differs markedly from it in that its molecular weight is 10 times greater than that of CPB. The serum enzyme may represent an aggregate of several subunits or it may be complexed with a nonenzymatic carrier protein. More work is needed to distinguish between these alternatives.

The question of whether AI requires activation from a proenzyme is of considerable biologic significance. It was found in active form present in all serum samples tested. Full activity was also present in plasma of freshly drawn heparinized blood. Storage of serum or plasma did not noticeably increase AI activity. These observations tend to support the view that AI occurs in the circulation as an active enzyme. If this view were correct, basic amino acid residues should not be found in C-terminal position of plasma proteins. In fact, none of the plasma proteins have been reported so far to possess C-terminal arginine or lysine (11).

A CPB-like enzyme has previously been shown to occur in serum of various species by Erdös (13). Although not yet purified, it has been characterized with respect to several properties. It is contained in Cohn fraction IV-1 of human serum, is eluted late from DEAE-Sephadex and emerges from Sephadex G-200 columns between the first and second peak of the serum profile (14). The enzyme inactivates bradykinin by removing the C-terminal arginine, and it hydrolyzes hippuryl-L-arginine and hippuryl-L-lysine. It is inactivated by EDTA and phenanthroline. It is probable that Erdös' enzyme, also called kininase I or carboxypeptidase N, is identical with the here described AI.

Elucidation of the effect of AI on C3a has revealed the essential role of the C-terminal group of C3a for its anaphylatoxin activity. It has further afforded identification of the tryptic peptide which contains the critical arginine residue. This peptide was found to be the most

positively charged of all peptides obtained from C3a by tryptic digestion.

The enzyme specificity of AI and its demonstrated ability to inactivate C5a anaphylatoxin indicate that a basic residue occupies also the C-terminus of this peptide, although C-terminal analyses have not yet been performed. Similarly, the effect of AI on C3a produced by the C3 inactivator complex<sup>8</sup> indicates a basic C-terminal residue for this fragment. It is most probable that its C-terminal group constitutes arginine, since arginine was found in this position in C3a produced either by trypsin or C4,2. According to preliminary reports from this laboratory, C3a (C4,2) contained C-terminal leucine (15). After the detection of AI and the recognition of its chemical effect on C3a, these earlier results were reexamined. They could be explained by the finding of trace amounts of AI in some preparations of the C4,2 enzyme. When in subsequent experiments C4,2 was used which was devoid of AI activity, the resulting C3a contained C-terminal arginine.

In contrast to the human anaphylatoxins, the anaphylatoxins of rat and guinea pig serum are resistant to inactivation by autologous and heterologous AI. Lack of susceptibility may be due to a blocked carboxyl group of the C-terminal arginine, absence of a basic C-terminal, or dependence of the activity upon groups other than a basic C-terminal residue. In the case of pig anaphylatoxin, the C-terminal residue is probably amidated or blocked otherwise, since Stegemann, Bernhard, and O'Neil (16) failed to liberate any residue using a mixture of CPA and CPB.

The potential biological significance of AI is indicated by the following *in vitro* observations. AI efficiently abolishes the activity of both types of anaphylatoxin, C3a and C5a. It thereby interferes with their potential to release histamine from cellular elements, and thus with all histamine-dependent reactions caused by anaphylatoxin. AI also abolished the capacity of C3a to cause rat uterus contraction which was found to be independent of histamine and serotonin release. Although less efficiently, AI destroys the activity of bradykinin and lysyl-bradykinin, as evidenced by loss of smooth muscle contracting activity. Certainly, the failure of many attempts to produce anaphylatoxin in whole human serum is adequately explained by the occurrence of AI in all normal sera tested. While this well established fact tended to minimize the possible *in vivo* role of the anaphylatoxins in man, the description of this new regulatory principle has reemphasized the anaphylatoxins as mediators of specific and nonspecific inflammation. That C3a anaphylatoxin is highly active in man was shown recently by intradermal administration of C3a to healthy individuals. Nanogram amounts caused an imme-

diate wheel and flare formation of 30-60 min duration (2).\*

### ACKNOWLEDGMENTS

The technical assistance of Mrs. Heike Ötting is gratefully acknowledged.

This work was supported by U. S. Public Health Service Grant AI-07007, American Heart Association, Inc. Grant 68-666, and United States Atomic Energy Commission Contract AT(04-3)-730.

### REFERENCES

1. Müller-Eberhard, H. J. 1969. Complement. *Annu. Rev. Biochem.* 38: 389.
2. Lepow, I. H., W. Dias Da Silva, and R. A. Patrick. 1969. Biologically active cleavage products of components of complement. In *Cellular and Humoral Mechanisms in Anaphylaxis and Allergy*. H. Z. Movat, editor. S. Karger, Basel. 237.
3. Bokisch, V. A., H. J. Müller-Eberhard, and C. G. Cochrane. 1969. 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. *J. Exp. Med.* 129: 1109.
4. Cochrane, C. G., and H. J. Müller-Eberhard. 1968. The derivation of two distinct anaphylatoxin activities from the third and fifth components of human complement. *J. Exp. Med.* 127: 371.
5. Andrews, P. 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. *Biochem. J.* 96: 595.
6. Hedrick, J. L., and A. J. Smith. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc electrophoresis. *Arch. Biochem. Biophys.* 126: 155.
7. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121: 404.
8. Katz, A. M., W. J. Dreyer, and C. B. Anfinsen. 1959. Peptide separation by two-dimensional chromatography and electrophoresis. *J. Biol. Chem.* 234: 2897.
9. Hirs, C. H. W. 1967. Performic acid oxidation. In *Methods in Enzymology*. C. H. W. Hirs, editor. Academic Press Inc., New York. Vol. 11. 197.
10. Gray, W. R., and B. S. Hartley. 1963. A fluorescent end-group reagent for proteins and peptides. *Biochem. J.* 89: 59P.
11. Schultze, H. E., and J. F. Heremans. 1966. *Molecular Biology of Human Proteins*. Elsevier, Amsterdam. Vol. 1.
12. Polley, M. J., and H. J. Müller-Eberhard. 1968. The second component of human complement: its isolation, fragmentation by C'1 esterase and incorporation into C'3 convertase. *J. Exp. Med.* 128: 533.
13. Erdös, E. G. 1966. Hypotensive peptides: bradykinin, kallidin and eledoisin. *Advan. Pharmacol.* 4: 1.
14. Erdös, E. G. 1969. The kallikrein-kinin-kininase system. In *Cellular and Humoral Mechanisms in Anaphylaxis and Allergy*. H. Z. Movat, editor. S. Karger, Basel. 233.
15. Budzko, D. B., and H. J. Müller-Eberhard. 1969. Liberation of anaphylatoxin by chemical cleavage of C3. *Fed. Proc.* 28: 817.
16. Stegemann, H., G. Bernhard, and J. A. O'Neil. 1964. Endgruppen von Anaphylatoxin. Einfache Sequenzanalyse von Carboxyl-endständigen Aminosäuren. *Z. Physiol. Chem.* 339: 9.