A Fragment of the Third Component of Human Complement with Anaphylatoxin Activity*

Delia B. Budzko, Viktor A. Bokisch,† and Hans J. Müller-Eberhard‡

ABSTRACT: A fragment (C3a) with anaphylatoxin activity was cleaved from the isolated third component of human complement (C3) either by the complement enzyme C3 convertase, trypsin, or mild treatment with hydroxylamine. The enzymatically produced C3a fragments had a molecular weight of approximately 7200, contained N-terminal serine, and C-terminal arginine, and had a virtually identical amino acid composition. C3a was found to be a very basic peptide, having an electrophoretic mobility at pH 8.5 of $+2.1 \times 10^{-5}$ cm^2 V^{-1} sec^{-1} and a ratio of basic to acid amino acid residues of 1.33. The hydroxylamine produced anaphylatoxin had a molecular weight of approximately 7800 and an amino acid composition resembling that of the enzymatically produced anaphylatoxin, except that it contained several additional residues, including glutamic acid, glycine, leucine, and arginine. The precursor, C3, had a molecular weight of 185,000, an electrophoretic mobility at pH 8.5 of -2.9×10^{-5} cm^2 V^{-1} sec^{-1}, and a ratio of basic to acid amino acid residues of 0.54. Serine was detected in N-terminal position and neither hydrazinolysis nor treatment with carboxypeptidases A and B revealed C-terminal residues. These results indicate that C3a anaphylatoxin corresponds to an unusually basic region of its precursor and suggest that it is derived from the N-terminal portion of C3. The hydroxylamine-susceptible bond appears to be located, relative to the enzymatically affected bond, further toward the C-terminal end of C3.

Anaphylatoxin is a reaction product of serum complement which has the capacity to contract smooth muscle and to cause increased capillary permeability. Most of the effects of anaphylatoxin are due to its ability to release histamine from cellular elements. Two chemically and biologically distinct anaphylatoxins have been described, one being a fragment of the third, the other a fragment of the fifth component of complement (Müller-Eberhard, 1969). These fragments arise in the complement reaction as a result of the action of indigenous complement enzymes. Thus, C3[1] derived anaphylatoxin (C3a) is generated by the enzyme $C\overline{4,2}$, which constitutes a fusion product of C2 and C4 formed by the action of $C\overline{1}$. Similar fragments with anaphylatoxin activity have been produced from C3 by the action of trypsin, plasmin and C3 inactivator complex (Bokisch *et al.*, 1969; Cochrane and Müller-Eberhard, 1968). C3a was recently isolated and its relationship to the precursor, C3, was characterized immunochemically (Bokisch *et al.*, 1969). The present study was initiated to obtain information on the chemical properties of C3a and its structural relationship to native C3. In the course of this work it was found that a C3a-like, biologically active fragment may be produced by mild treatment of C3 with hydroxylamine (Budzko and Müller-Eberhard, 1969) and a comparative analysis of this chemically obtained piece with the enzymatically derived fragment will also be presented.

Materials and Methods

C3 and C3 Fragments. Highly purified C3 was prepared from fresh human serum as described previously (Nilsson and Müller-Eberhard, 1965). Fragmentation of C3 by $C\overline{4,2}$ (Müller-Eberhard *et al.*, 1967) and trypsin was performed according to published methods (Bokisch *et al.*, 1969). In the course of this study it became apparent that some anaphylatoxin preparations were partially inactivated through the presence of trace amounts of a serum carboxypeptidase which contaminated the starting materials (Bokisch and Müller-Eberhard, 1971). Therefore all preparations of isolated C3 and all reagents used for the generation of the $C\overline{4,2}$ enzyme were examined for presence of the anaphylatoxin inactivator, and unless they were found to be free of this contaminant, they were omitted from further use. C3a and C3b were separated by filtration on Sephadex G-100 columns or by preparative polyacrylamide gel electrophoresis in an acid buffer system as described (Bokisch *et al.*, 1969). The C3a-like fragment produced by hydroxylamine treatment was obtained by the following method; 30 mg of isolated C3 in 10 ml of sodium phosphate buffer (pH 7.0), ionic strength 0.1, was mixed with 10 ml of 1 M hydroxylamine solution prepared according to Lipmann and Tuttle (1945). The mixture was held at 20° for 20 min and the reaction was then stopped by transferring the mixture to an ice bath and by increasing the hydrogen ion concentration with 1 N HCl to pH 3.8. The protein solution was dialyzed overnight at 4° against sodium acetate buffer (pH 4.0), ionic strength 0.1, using Visking tubing $^{23}/_{32}$ (Union Carbide, Chicago, Ill.). The protein was concentrated five times in an Amicon pressure filtration device using a Diaflo ultrafiltration membrane, series UM-2 (Amicon Corp., Lexington, Mass.), and applied to a 90 × 2.5 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) which was equilibrated with 0.01 M sodium acetate buffer

* This is publication number 403 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received March 30, 1970.* This work was supported by U. S. Public Health Service Grant AI-07007, American Heart Association, Inc., Grant 68-666, and U. S. Atomic Energy Commission Contract AT(04-3)-730.

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

‡ To whom to address correspondence.

[1] The symbols used have been recommended by the *W.H.O.* Committee on Complement Nomenclature (1968). C1, C2, C3, C4, and C5 denote, respectively, the first, second, third, fourth, and fifth components of the complement system. $C\overline{1}$ designates the enzymatically active form of C1 and $C\overline{4,2}$ the activating enzyme of C3, which is also called C3 convertase. C3a and C3b refer to cleavage products of C3 produced either by $C\overline{4,2}$ or trypsin (try). The enzyme employed is indicated in parenthesis following the symbol of the fragment, *e.g.*, C3a(try).

(pH 3.8), containing 0.15 M NaCl. The flow rate was 15 ml/hr and fractions of 3.5 ml were collected. The low molecular weight fragment was eluted in fractions immediately preceding the internal column volume. In two experiments the hydroxylamine produced, biologically active fragment was isolated by preparative gel electrophoresis in a Büchler apparatus (Büchler Instruments Co., Fort Lee, N. J.) using a 3.5-cm separating column of 6% polyacrylamide gel concentration and buffer of pH 4.5 as described by Reisfeld *et al.* (1962). A current of 250 mA was applied, the flow rate was adjusted to 25 ml/hr and fractions of 3 ml were collected. Under these conditions the C3a-like fragment was completely eluted after 3.5 hr and was contained in three fractions or 9 ml of effluent. The elution diagram is shown in Figure 10.

Molecular Weight Determinations. The polyacrylamide gel electrophoresis method of Hedrick and Smith (1968) was used. The gel concentration ranged from 5 to 12%. Glycine–Tris buffer, pH 8.3 (Davis, 1964), was employed for analysis of C3 and C3b and β-alanine–acetic acid buffer, pH 4.5 (Reisfeld *et al.*, 1962), for analysis of the C3a fragment. The relative distance of migration, R_m, of the proteins were determined using as reference substance bromophenol blue in the experiments performed at pH 8.3 and pyronine Y or the buffer interphase in those carried out at pH 4.5. The position of the markers was indicated prior to staining by inserting copper wires from the lower end of the gels. The logarithm of the R_m was graphically related to the gel concentration and the slope of this plot was calculated and graphically related to the molecular weight of reference proteins.

Amino Acid Analyses. The amino acid compositions of C3 and C3a were determined by the method of Spackman *et al.* (1958) using a Beckman-Spinco Model 120C amino acid analyzer equipped with an automatic sample injector and a Model 125 automatic digital integrator. The protein was hydrolyzed in constant-boiling or 6 N HCl for 18 hr at 105° in evacuated and sealed Pyrex tubes. Approximately 15 nmoles of hydrolyzed protein was applied to each column and the amino acid concentration determined by comparison with a standard mixture of amino acids (100 nmole/ml). Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation of the protein (Hirs, 1956). Free SH groups were quantitated as *S*-carboxymethylcysteine; 1 mg of C3a protein in 1 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 8 M urea was treated with 3.6×10^{-2} M iodoacetamide for 60 min at 20°. The solution was dialyzed extensively against 0.016 M sodium phosphate buffer (pH 6.5) and the protein was concentrated by lyophilization for subsequent hydrolysis.

Analysis of Amino-Terminal Residues. The fluorodinitrobenzene (FDNB)[2] method of Porter and Sanger (1948) was used. Dinitrophenylation of C3 and C3b was performed on 0.1 μmole of protein in 1.5% $NaHCO_3$ solution containing 8 M urea or 5 M guanidine hydrochloride. Usually the dinitrophenyl (DNP) derivative was hydrolyzed in 6 N HCl for 16 hr at 105°. To analyze for DNP-glycine, hydrolysis was limited in two separate experiments to 4.5 hr and to analyze for DNP-proline, hydrolysis was carried out in 11 N HCl for 5 hr at 105°. In one experiment C3 was oxidized with performic acid prior to dinitrophenylation (Hirs, 1956). C3a preparation (0.1 μmole) was treated with FDNB in 1% aqueous trimethylamine in absence of urea or guanidine hydrochloride.

DNP-amino acids were identified by one-dimensional paper chromatography (Fraenkel-Conrat *et al.*, 1955) or by two-dimensional silica gel thin-layer chromatography using five different solvent systems described by Brenner *et al.* (1961). The DNP-amino acids were quantitated spectrophotometrically (Levy, 1954), after elution from paper with water at 56° and from silica gel with 0.05 M Tris buffer (pH 8.6).

N-Terminal analysis of C3a by dansylation was carried out according to Gray and Hartley (1963) and separation of the dansylamino acids was performed by polyamide-layer chromatography (Woods and Wang, 1967).

Analysis of Carboxy-Terminal Residues. DFP-treated carboxypeptidases A and B (COADFP 8FB, COBDFP 61A, Worthington Biochemical Corp., Freehold, N. J.), separately or in mixture, were used for C-terminal analysis of C3, C3a, and C3b. In several experiments C3 was either reduced and alkylated or succinylated prior to treatment with the enzymes. C3 (50 nmoles) in 5 ml of Tris buffer (pH 8.6) was treated for 1 hr at 20° with 2×10^{-3} M dithiothreitol. Iodoacetamide was then added to a final concentration of 2.2×10^{-3} M and the mixture was kept at 20° for 15 min before it was exhaustively dialyzed. For succinylation, an identical amount of C3 in the same buffer was used and 5 μmole of succinic anhydride was added in small increments and the pH was maintained at 7.5 by addition of 1 N NaOH. The succinylated C3 was then dialyzed for treatment with the enzymes. Approximately 50 nmoles of protein was treated in 1 M ammonium bicarbonate buffer (pH 8.1) with enzyme (molar ratio 20:1) for periods of 15 min to 2 hr at room temperature. Treatment of C3 and C3b was stopped by addition of 20 volumes of absolute alcohol. After removal of the protein precipitate by centrifugation, the supernatant was evaporated to dryness under vacuum at 50° and the residue derived from 15 to 20 nmoles of treated protein was subjected to amino acid analysis. Treatment of C3a was stopped by decreasing the pH to 3.0 with 1 N HCl, and the reaction mixture was passed over a 2-ml column of packed Dowex AG 50W-X8 (H+), 200–400 mesh. The protein was eliminated by washing the resin with 5 ml of distilled water and the amino acids were then eluted with 2 ml of 3 M ammonium hydroxide. After evaporation of the solvent the residue corresponding to 15–20 nmoles of treated protein was analyzed for liberated amino acids. Recovery of arginine and of norleucine from the Dowex column was, respectively, 65 and 90%. The experimental values were therefore corrected accordingly.

Hydrazinolysis was performed according to Akabori *et al.* (1952) and the free amino acids were analyzed as described above.

Electrophoresis. Analytical electrophoresis on cellulose acetate strips was carried out in a Beckman microzone electrophoresis apparatus, Model R-101, using Beckman barbital buffer (pH 8.5), ionic strength 0.075, and 250 V for 20 min at 20°.

Analytical polyacrylamide gel electrophoresis was performed according to Reisfeld *et al.* (1962) using 6% resolving gel and β-alanine–acetic acid buffer (pH 4.5) for demonstration of C3a, and Tris-HCl buffer, pH 8.7 (Davis, 1964), for analysis of native C3.

Analytical Ultracentrifugation. Native C3 was examined at 7.4 mg/ml in phosphate buffer (pH 7.0), ionic strength 0.1. Isolated C3a was analyzed at 5.8 mg/ml in 0.15 M acetate-buffered sodium chloride (pH 3.6). The experiments were performed in a Spinco Model E machine at 20° and 52,640 rpm.

Assay of Anaphylatoxin Activity. Segments of guinea pig ileum were used for this assay and contractions were measured as described (Cochrane and Müller-Eberhard, 1968).

[2] Abbreviation used is: FDNB, fluorodinitrobenzene.

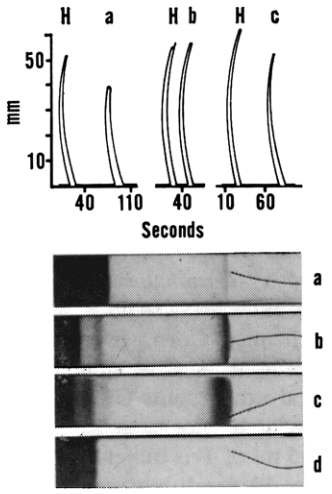

FIGURE 1: Fragmentation of C3 by three different methods and concomitant generation of anaphylatoxin activity. The upper panel shows recordings of the contraction of guinea pig ileum caused by C3 treated with hydroxylamine (a), trypsin (b), and C3 convertase (c). Untreated C3 did not cause any contraction (not shown). H designates contractions produced with 0.2 μg of histamine. The lower panel shows acid disc electrophoresis analysis of the three treated C3 preparations (a, b, and c) and the untreated control (d). Protein (150–300 μg) was applied; the top of the gels is at the left and the cathode was at the right. The wire tips indicate the position of the marker. The protein band located near the marker position represents C3a, the low molecular weight, biologically active split product of C3.

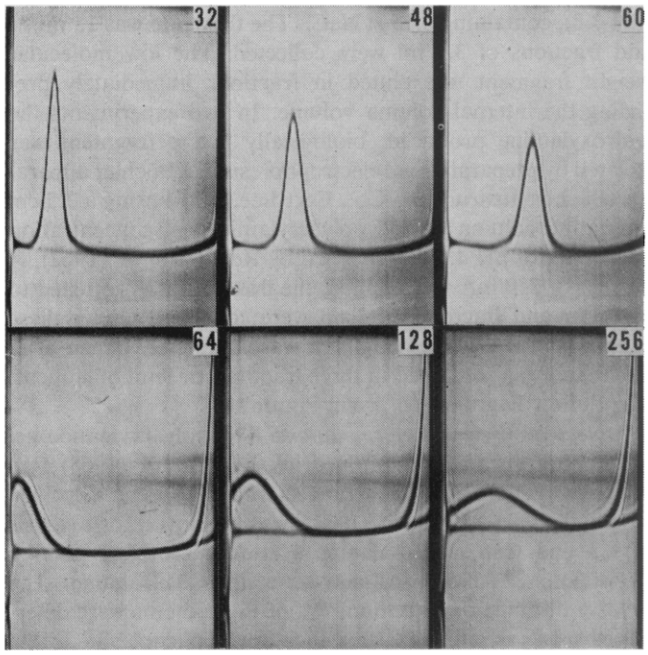

FIGURE 3: Ultracentrifugal analysis of isolated C3 and isolated C3a. C3 (upper frames) was examined at 7.4 mg/ml in phosphate buffer, pH 7.0, ionic strength 0.1. C3a (lower frames) was examined at 5.8 mg/ml in 0.15 M acetate buffered NaCl, pH 3.6. The speed in both experiments was 52,640 rpm the temperature 20°; the numbers indicate time in minutes of centrifugation.

Results

Cleavage of C3a from Isolated C3 and Concomitant Generation of Anaphylatoxin Activity. Three methods were used in this study for the fragmentation of C3: treatment with trypsin (1%, w/w) for 1 min at 20°; treatment with the complement enzyme $C\overline{4,2}$ (1%, w/w) for 20 min at 37°; and treatment with hydroxylamine (0.5 M, 20 min 20°). The appearance of a low molecular weight fragment may be demonstrated by acid disc electrophoresis as shown in Figure 1. This figure also contains records of the anaphylatoxin activity which was generated concomitant with dissociation of C3a from its precursor. Treatment of C3 with 1 M KBr for 5 hr at 4°, or with 1% sodium dodecyl sulfate for 2 hr at 20°, did not result in appearance of C3a. C3a was separated from the C3b fragment by filtration through Sephadex G-100, it was then concentrated and passed a second time over a Sephadex G-100 column. Both molecular sieve chromatograms are depicted in Figure 2. The C3a fragments were biologically active in their isolated form, approximately 2 μg being sufficient to contract a segment of guinea pig ileum in a 20-ml bath. The activity was inhibitable in each instance with antihistamine (2 μg of chlortrimeton, Schering Corp., Bloomfield, N. J.).

Ultracentrifugal and Electrophoretic Analysis of Isolated C3a and Its Precursor. The homogeneity of the isolated proteins which were subjected to chemical analyses was ascertained by

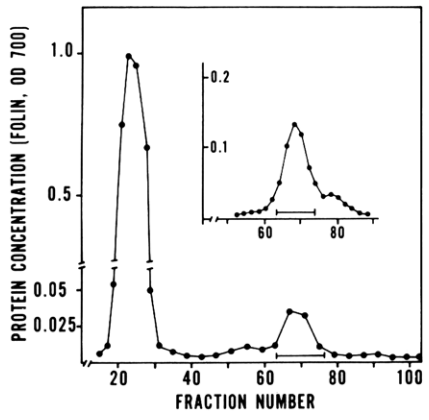

FIGURE 2: Isolation of C3a by molecular sieve chromatography. After treatment of C3 with C3 convertase, the resulting fragments were separated by filtration through a 2.5 × 90 cm column of Sephadex G-100 which was equilibrated with 0.01 M sodium acetate buffer (pH 3.8) containing 0.15 M NaCl. The material contained in fractions 62–78 was concentrated and rechromatographed on the same column (insert). The horizontal bar indicates the fractions pooled for further analysis.

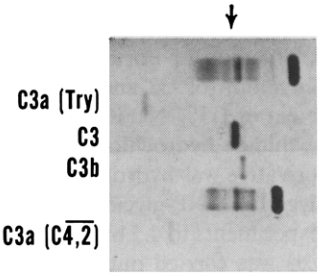

FIGURE 4: Zone electrophoretic analysis of C3 and its fragments, demonstrating the very basic nature of C3a(try) and C3a($C\overline{4,2}$). The arrow indicates the origin, the cathode was to the left, cellulose acetate strips were used as supporting medium and the pH was 8.5. Since the figure is a composite of two separate strips which were not strictly comparable, whole human serum was included on each for reference purposes. The electrophoretic mobility was $+2.1 \times 10^{-5}$ cm^2 V^{-1} sec^{-1} for trypsin and C4,2 produced C3a. One microliter was applied containing 5–10 mg of isolated protein/ml.

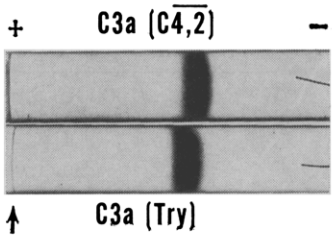

FIGURE 5: Disc electrophoresis of isolated C3a produced by C4,2 or trypsin. Electrophoresis was performed in gels of 10% polyacrylamide in β-alanine–acetic acid buffer (pH 4.5). The top of the gels is at the left, the tips of wires inserted from the cathodal end of the gels indicated position of buffer interface; 40 μg of protein was applied.

analytical ultracentrifugation (Figure 3), electrophoresis on cellulose acetate strips at pH 8.5 (Figure 4), and by disc electrophoresis at pH 4.5 on 10% polyacrylamide gels (Figure 5). By comparison to the known electrophoretic mobilities of albumin and γG-globulin in whole human serum, the mobilities of C3 and its fragments, C3a and C3b, were estimated to be, respectively, -2.9×10^{-5}, $+2.1 \times 10^{-5}$, and -3.2×10^{-5} $cm^2\ V^{-1}\ sec^{-1}$.

Molecular Weights of C3 and Its Fragments. The relative electrophoretic mobility of C3, C3a, and C3b in gels of various polyacrylamide concentrations is shown in Figure 6. In all

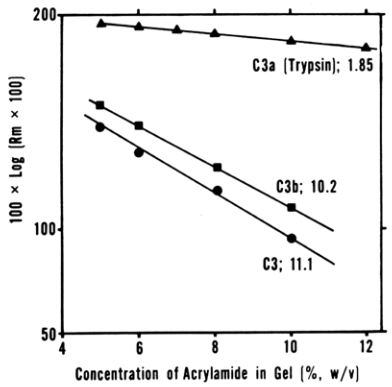

FIGURE 6: Molecular weight determination of C3 and the C3 fragments by the polyacrylamide gel electrophoresis method. The slopes of the R_m *vs.* gel concentration plots are indicated by the numbers. C3a was analyzed at pH 4.5, C3 and C3b at pH 8.6.

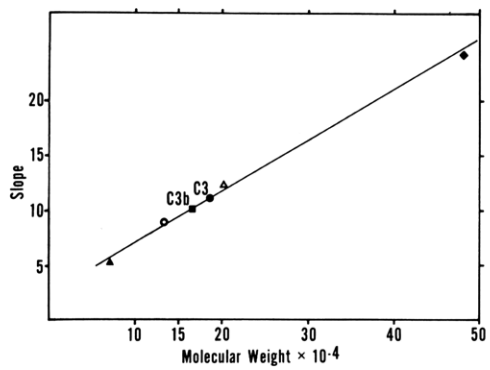

FIGURE 7: Molecular weight determination of C3 and C3b from the slopes of the R_m *vs.* gel concentration plots (Figure 4). As reference substances were used human albumin monomer (▲), dimer (○), trimer (△), and urease (◆).

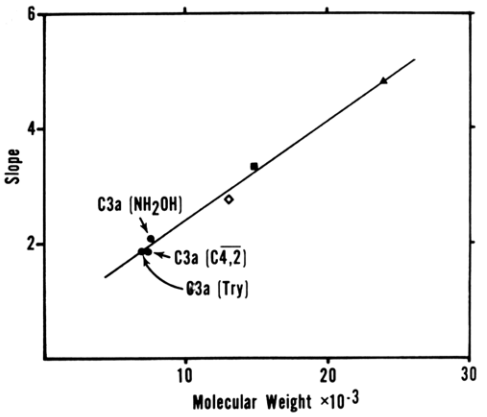

FIGURE 8: Molecular weight determination of the C3a fragments from the slopes of the R_m *vs.* gel concentration plots. As reference substances were used lysozyme (◇), C4a, the low molecular weight cleavage product of C4 (mol wt 15,000) (Budzko and Müller-Eberhard, 1970) (■), and trypsin (▲).

gels only one disk was observed with each of the three proteins examined. The molecular weights of C3 and C3b were obtained from the plot depicted in Figure 7, and those of C3a(C4,2), C3a(try) and C3a(NH_2OH) from the plot shown in Figure 8. The results are listed in Table I. The minimum molecular weights of the low molecular weight fragments which were calculated from their histidine and tyrosine content are also listed in Table I. From these data the mean molecular weight values were calculated to be 7200 for C3a(try) and C3a(C4,2) and 7800 for C3a(NH_2OH).

Amino Acid Composition of the Enzymatically Produced C3a Fragments and Their Precursor. The results of replicate amino acid analyses are listed in Table II. The concentration of basic residues is more than two times greater in the biologically active C3a fragments than in native C3, and the concentration of acid residues is somewhat lower in C3a than in the precursor. In addition, the concentration of half-cystine in anaphylatoxin is more than three times that of the precursor. A comparison of C3a(try) and C3a(C4,2) revealed a high degree of similarity. The calculations of the number of residues per molecule of C3a were based on mean molecular weight values (see above).

N- and C-Terminal Residues. The results of the analysis of N-terminal residues by the dinitrophenyl method are summarized in Table III. Serine was the only detectable N-ter-

TABLE I: Molecular Weights of C3 and C3 Fragments.

Protein	Molecular Weight: Gel Electrophoresis Method	Molecular Weight: Amino Acid Anal.[a]
C3	185,000	
C3b	165,000	
C3a(C4,2)	7,500	6992
C3a(try)	7,500	6914
C3a(NH_2OH)	8,600	7000

[a] Based on tyrosine and histidine content determined by three separate analyses.

TABLE II: Amino Acid Composition of C3 and Its Biologically Active Fragments.

	Moles Amino Acid/Mole of Protein[a]				Residues/1000 Residues			
	C3	C3a ($C\overline{4,2}$)	C3a (try)	C3a (NH_2OH)	C3	C3a ($C\overline{4,2}$)	C3a (try)	C3a (NH_2OH)
Lysine	100.1[b]	4.8	4.8	4.8	68.3	85.0	85.0	74.3
Histidine	25.0	1.4	1.1	1.5	17.1	24.8	19.5	23.2
Arginine	63.2	7.5	6.9	8.7	43.2	132.8	122.1	134.6
Aspartic acid	143.3	4.1	4.6	4.7	97.8	72.6	81.4	72.7
Threonine	92.1	2.7	3.1	3.0	62.9	47.8	54.9	46.4
Serine	94.0	3.1	3.4	4.0	64.2	54.9	60.2	61.9
Glutamic acid	203.8	6.4	6.3	7.2	139.1	113.3	111.5	111.4
Proline	73.1	2.9	2.9	2.3	49.9	51.3	51.3	35.6
Glycine	92.6	3.2	3.1	5.9	63.2	56.6	54.9	91.3
Alanine	87.1	2.7	2.5	3.2	59.5	47.8	44.3	49.5
Half-cystine	34.0	3.7	4.1	ND[c]	23.2	65.5	72.6	60.3
Valine	129.6	2.2	2.5	2.8	88.5	38.9	44.3	43.3
Methionine	25.8	1.9	1.8	ND	17.6	33.6	31.9	28.6
Isoleucine	63.6	1.4	2.0	1.8	43.4	24.8	35.4	27.8
Leucine	132.1	5.3	4.1	5.6	90.2	93.8	72.6	86.6
Tyrosine	51.2	1.2	1.2	1.1	35.0	21.2	21.2	17.0
Phenylalanine	54.0	2.0	2.2	2.3	36.9	35.4	38.9	35.6
Total no. of residues	1464.6	56.5	56.6	64.7	1000.0	1000.1	1001.8	1000.1

[a] Calculated for molecular weights of 185,000, C3; 7,200, C3a($C\overline{4,2}$); 7,200, C3a(try); 7,800 C3a(NH_2OH). [b] Numbers are averages of separate experiments: 3 for C3, 5 for C3a($C\overline{4,2}$), 10 for C3a(try) and 2 for C3a(NH_2OH). [c] Not determined; for calculation of total number of residues of C3a(NH_2OH), the mean of the values for C3a($C\overline{4,2}$) and C3a(try) were used.

minal residue in native C3 and in C3a, whereas aspartic acid, glutamic acid and serine were detected in the C3b fragment. Serine was also found in N-terminal position of C3a using the dansylation method. The results of C-terminal residue analysis are listed in Table IV. A C-terminal residue was neither detectable in C3 nor in C3b. Treatment of C3 with carboxypeptidases A and B was performed (a) for 1–24 hr at 20° and pH 8.0, (b) at different hydrogen ion concentrations between pH 7.0 and 9.5, (c) after heating of C3 at 65° for 30 min in phosphate buffer, pH 7.0, (d) after reduction and alkylation of the protein, (e) after complete succinylation, and (f) in 6 M urea. Under none of these conditions could release of amino acids be observed. Further, hydrazinolysis also failed to indicate a C-terminal residue of C3. Kinetic analysis of release of amino acids by carboxypeptidases A and B from C3a(try) suggested that arginine constitutes the C-terminal residue and

TABLE III: N-Terminal Amino Acids of C3 and Its Fragments Determined by the Dinitrophenyl Method.

Protein	DNP-amino Acid	Moles of DNP-amino Acid/Mole of Protein			
		1	2	3	Av
C3	Serine	0.7	1.1	1.3	1.1
C3a(try)	Serine	0.5			0.5
C3a($C\overline{4,2}$)	Serine	0.23	0.17		0.2
C3b(try)	Aspartic acid	0.3	0.42		0.36
	Glutamic acid	0.42	0.45		0.44
	Serine	0.61	0.38		0.49
C3b($C\overline{4,2}$)	Aspartic acid	0.26	0.42		0.34
	Glutamic acid	0.37	0.39		0.38
	Serine	0.26	0.52		0.39

TABLE IV: C-Terminal Amino Acids of C3 and Its Fragments Determined Using Carboxypeptidases A and B.

Protein	Carboxy-peptidase	Amino Acid	
		Residue	Moles/Mole of Protein
C3	A + B	None detectable	
C3a($C\overline{4,2}$)	B	Arginine	0.64 (120 min)
	A (after B)	Leucine	0.79 (120 min)
		Alanine	0.70 (120 min)
	A + B	Arginine	0.62 (90 min)
		Leucine	1.0 (90 min)
		Alanine	0.74 (90 min)
C3a(try)	B	Arginine	0.67 (20 min), 0.98 (60 min)
	A + B	Arginine	1.45 (60 min), 1.63 (120 min)
		Leucine	0.24 (60 min), 0.30 (120 min)
		Alanine	0.39 (60 min), 0.45 (120 min)
C3b(try)	A + B	None detectable	

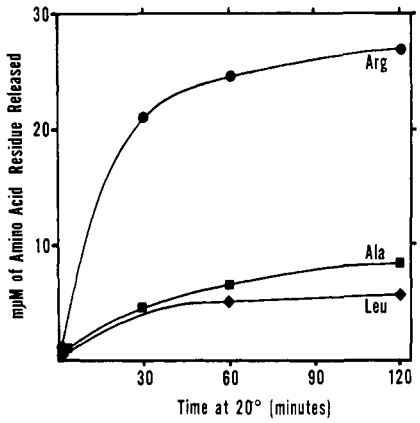

FIGURE 9: Kinetic analysis of the release of amino acids from C3a-(try) by carboxypeptidases A and B. Each point in the graph represents the analysis of 16.6 mμmoles.

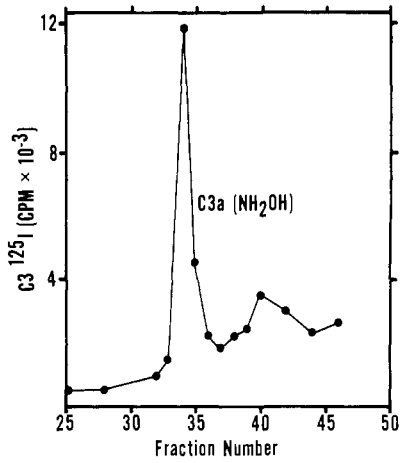

FIGURE 10: Isolation of C3a(NH_2OH) by preparative polyacrylamide gel electrophoresis. NH_2OH-treated C3 (30 mg) was applied, the pH was 4.5. Fractions 34–36 were pooled and concentrated for further analysis.

that alanine and leucine are released subsequently (Figure 9). Treatment of C3a($C\overline{4,2}$) with carboxypeptidase B alone liberated arginine in amounts approaching one mole per mole of protein. Subsequent treatment with carboxypeptidase A released alanine and leucine (Table IV). It may be concluded that both active C3a fragments contain arginine in the C-terminal position.

Analysis of Anaphylatoxin for Free Sulfhydryl Groups. Since there were approximately four half-cystine residues per molecule of C3 anaphylatoxin, the presence of free sulfhydryls was investigated. C3a(try) and C3a($C\overline{4,2}$) after treatment with iodoacetamide contained no detectable *S*-carboxymethylcysteine.

Characterization of Anaphylatoxin Released from C3 by Hydroxylamine. C3a(NH_2OH) was isolated after treatment of C3 with 0.5 M NH_2OH for 20 min at 20° by preparative polyacrylamide gel electrophoresis as shown in Figure 10. By the gel electrophoresis method (Figure 8, Table I), the molecular weight was 8600, the minimum molecular weight calculated from the tyrosine content was 7000. The amino acid composition resembled that of the enzymatically produced C3 anaphylatoxins (Table II), except that C3a(NH_2OH) contained in addition approximately one residue of arginine, two of glycine, and possibly one of glutamic acid and one of serine. The presence of additional amino acid residues is in agreement with the somewhat larger molecular weight (average value 7800) of this piece. The N- and C-terminal residues have not been determined.

Discussion

A study of C3-derived anaphylatoxin (C3a) and its relation to its precursor was initiated because C3a is a naturally occurring protein fragment of relatively small molecular size which is amenable to structural analysis. Its phlogogenic activity is biologically significant (Cochrane and Müller-Eberhard, 1968; Dias Da Silva *et al.*, 1967).

The data indicate that C3a is derived from a very basic portion of the precursor molecule, C3. At pH 8.6 it has a strongly positive net charge, whereas the precursor is negatively charged. Comparison of the trypsin- and the $C\overline{4,2}$-produced anaphylatoxins revealed a high degree of similarity. Their size was indistinguishable by the methods employed and replicate amino acid analyses showed only minor differences. Both fragments contained serine in N-terminal position, although the amount of serine measured was less than one mole of protein. At the C-terminal end the two anaphylatoxins contained arginine in amounts approaching one mole per mole of protein.

The amount of C-terminal arginine liberated by carboxypeptidase B from some preparations of C3a($C\overline{4,2}$) was considerably below one mole per mole of fragment. These preparations showed a reduced biological activity. This finding could be explained by trace contamination of some batches of C3 convertase with a serum carboxypeptidase which efficiently inactivates anaphylatoxin by removing the C-terminal arginine. The anaphylatoxin-inactivating enzyme of human plasma was subsequently isolated and characterized (Bokisch and Müller-Eberhard, 1971).

Stegemann *et al.* (1964, 1965) purified anaphylatoxin from dextran or cobra venom treated rat and hog plasma. Molecular weight estimates ranged from 2000 to 30,000, N-terminal residue analysis yielded arginine, and although mixtures of carboxypeptidases A and B abolished the biologic activity, C-terminal residues could not be detected. A comparison of the amino acid composition of rat and hog anaphylatoxin with that of human C3a underscores the chemical dissimilarity existing between C3a and the anaphylatoxin of these two animal species.

On the basis of the available data it may be postulated that the biologically active fragment C3a is cleaved from the N-terminal portion of C3. This view is supported by the finding of N-terminal serine in both C3 and C3a, by the demonstration of C-terminal arginine in C3a and the failure to detect this or any other C-terminal group in C3 or C3b. According to this hypothesis C3b should contain an N-terminal group which is not present in the precursor molecule. Why, instead, three different N-terminal residues were found in C3b cannot be explained at present.

The overall similarity of C3a($C\overline{4,2}$) and C3a(try) indicates that C3 convertase and trypsin attack the same region of native C3, and possibly the same bond. This bond should involve the carboxyl group of an arginine residue (C-terminal residue of C3a). That this region of native C3 is exceedingly susceptible to enzymatic attack is illustrated by the effect of trypsin: cleavage of C3 by trypsin (1%, w/w) into C3a and C3b is completed in 60 sec at 20°. It is also effected by plasmin, thrombin, and the so-called C3 inactivator complex (Bokisch

et al., 1969). This attack region of C3 is essential for the cytolytic activity of the protein. C3 is cytolytically inactive in its native form and requires activation in order to participate in the complement reaction. Its physiological activating enzyme is C3 convertase (Müller-Eberhard *et al.*, 1967), action of which on C3 enables it to bind to cell membrane surface receptors or to groups present on antibody molecules (Müller-Eberhard *et al.*, 1966). Thus, the reaction in which C3a is cleaved from C3 by C3 convertase also reveals the combining site in the residual, larger portion of the C3 molecule.

That the attack region of C3 has uncommon chemical properties is shown by its susceptibility to mild treatment with hydroxylamine. The C3a-like fragment with anaphylatoxin activity obtained with hydroxylamine was slightly larger than that of enzymatically produced C3a and contained a few more amino acid residues than C3a. In terms of the model proposed for the derivation of enzymatically produced anaphylatoxin, hydroxylamine appears to liberate essentially the same fragment. However, the bond cleaved by hydroxylamine appears to be situated several residues further toward the C-terminal end than the enzymatically susceptible bond.

Hydroxylamine-susceptible bonds have been shown to occur in some proteins, notably in collagen (Piez, 1968). They have been considered ester or ester-like bonds. However, recently one of the hydroxylamine-sensitive bonds of collagen has been shown to be a peptide bond linking an asparagine and a glycine residue (Butler, 1969). More work is needed to determine the nature of the hydroxylamine-sensitive bond in C3.

References

Akabori, S., Ohno, K., and Narita, K. (1952), *Bull. Chem. Soc. Jap. 25*, 214.

Bokisch, V. A., and Müller-Eberhard, H. J. (1971), *J. Clin. Invest. 49*, 2427.

Bokisch, V. A., Müller-Eberhard, H. J., and Cochrane, C. G. (1969), *J. Exp. Med. 129*, 1109.

Brenner, M., Niederwieser, A., and Pataki, G. (1961), *Experientia 17*, 145.

Budzko, D. B., and Müller-Eberhard, H. J. (1969), *Science 165*, 506.

Budzko, B. D., and Müller-Eberhard, H. J. (1970), *Immunochemistry 7*, 227.

Butler, W. T. (1969), *J. Biol. Chem. 244*, 3415.

Cochrane, C. G., and Müller-Eberhard, H. J. (1968), *J. Exp. Med. 127*, 371.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 125*, 404.

Dias Da Silva, W., Eisele, J. W., and Lepow, I. H. (1967), *J. Exp. Med. 126*, 1027.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 361.

Gray, W. R., and Hartley, B. S. (1963), *Biochem. J. 83*, 599.

Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Levy, A. L. (1954), *Nature (London) 174*, 126.

Lipmann, F., and Tuttle, L. C. (1945), *J. Biol. Chem. 159*, 21.

Müller-Eberhard, H. J. (1969), *Annu. Rev. Biochem. 38*, 389.

Müller-Eberhard, H. J., Dalmasso, A. P., and Calcott, M. A. (1966), *J. Exp. Med. 123*, 33.

Müller-Eberhard, H. J., Polley, M. J., and Calcott, M. A. (1967), *J. Exp. Med. 125*, 359.

Nilsson, U. R., and Müller-Eberhard, H. J. (1965), *J. Exp. Med. 122*, 377.

Nomenclature of Complement (1968), *Bull. W. H. O. 39*, 935.

Piez, K. A. (1968), *Annu. Rev. Biochem. 37*, 547.

Porter, R. R., and Sanger, F. (1948), *Biochem. J. 42*, 287.

Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Stegemann, H., Bernhard, G., and O'Neil, J. (1964), *Z. Phys. Chem. (Frankfurt am Main) 339*, 9.

Stegemann, H., Hillebrecht, R., and Rien, W. (1965), *Z. Phys. Chem. (Frankfurt am Main) 340*, 11.

Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133*, 369.