COMPLEMENT AS A MEDIATOR OF INFLAMMATION

III. Purification of the Activity with Anaphylatoxin Properties Generated by Interaction of the First Four Components of Complement and its Identification as a Cleavage Product of C'3*

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Interaction in free solution of purified preparations of the first four reacting components of human complement (C'1 esterase, C'4, C'2, and C'3)[1] in the presence of Mg^{2+} results in generation of a biological activity which satisfies criteria for designation as anaphylatoxin (1). The biological properties of such mixtures include contraction of guinea pig ileum with tachyphylaxis, failure to contract rat uterus, enhancement of vascular permeability in guinea pig skin, degranulation of mast cells in guinea pig mesentery preparations, and release of histamine from suspensions of rat peritoneal mast cells. These observations were an outgrowth of earlier studies on the role of C'1 esterase as a permeability factor (2), and in the generation of anaphylatoxin activity from fresh guinea pig or rat serum (3, 4).

The possibility that anaphylatoxin emerges as a reaction product at, or shortly after, completion of the C'3 step in the complement sequence was suggested by previous experiments based on such indirect criteria as a require-

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[1]The complement system, as presented and discussed in this paper, comprises 11 serum proteins. C'1 is a macromolecular complex of three proteins, C'1q, C'1r, and C'1s. The enzymatically active subunit derived from C'1s is referred to as C'1 esterase. Subsequently acting components, in the order of their reaction, are C'4, C'2, C'3, C'5, C'6, C'7, C'8, and C'9.

ment for Mg^{2+} and inhibition by salicylaldoxime and phlorizin (4). C'3 was further implicated as a component from which anaphylatoxin might be derived by the findings that biologically active mixtures could be prepared from human C'1 esterase, C'4, C'2, and C'3 without known participation of later acting components; that the muscle-contracting activity of such mixtures was associated with fractions of lower molecular weight than any of the reactants; and that appearance of biological activity was closely correlated with immunoelectrophoretic conversion of C'3 to anodically faster components (1, 5). Through the use of labeled, purified components of human complement and by separation of the active product by gel filtration or sucrose density gradient ultracentrifugation, we have now shown that generation of anaphylatoxin from mixtures of C'1 esterase, C'4, C'2, and C'3 is a direct consequence of cleavage of C'3, yielding a low molecular weight split product which possesses all of the biological properties of the original mixture. It is the purpose of this paper to present evidence supporting this conclusion and to describe some characteristics of the reaction mechanism and of the isolated product.

Materials and Methods

Preparation, Assay, and Labeling of Human Complement Components

Serum.—Blood was collected without anticoagulant from apparently healthy human donors and allowed to clot at room temperature for 1–2 hr and at 2°C for several additional hours or overnight. Serum was separated by centrifugation at 2°C. Pools of serum from 2-25 donors were either used immediately or stored at −60°C.

C'1 Esterase, C'4, C'2, and C'3.—These components were prepared, assayed and stored by modifications of published procedures (6–9), exactly as described in a previous paper (1).

C'5.—This component was prepared as a by-product of C'3 purification, as described by Nilsson and Müller-Eberhard (9). Functional activity was measured by restoration of the hemolytic activity of human serum treated with M potassium thiocyanate (KSCN), in the presence of purified C'3 and C'4 (10). Preparations of C'5 were free from immunologically detectable C'3 and C'4. They were either used fresh or quick-frozen in dry ice–acetone and stored at −60°C. A single preparation of highly purified C'5 was also kindly donated by Dr. H. J. Müller-Eberhard and gave results comparable to those obtained with C'5 made in this laboratory.

Labeling of Complement Components.—C'1 esterase, C'4, C'2, C'3, and C'5 were labeled with ^{125}I or ^{131}I. The results were independent of the iostope used; only experiments with ^{125}I will be reported here. Labeling was performed in pH 8.0 borate buffer by the iodine monochloride method of Helmkamp and coworkers (11) at a tagging efficiency ranging from 40–80%. The extent of labeling did not exceed 1 atom of iodine per molecule of protein. Unbound iodine was removed by exhaustive dialysis against 0.15 M NaCl and then PBS buffer (pH 7.4 phosphate-buffered saline, 5×10^{-4} M Mg^{2+}) at 2°C, followed by concentration of the labeled protein by ultrafiltration and either passage through Biogel P-60 or ultracentrifugation in 5–20% sucrose density gradients. The final preparations, in PBS buffer, were either used directly or first diluted with unlabeled component to reduce the amount of radioactivity introduced into reaction mixtures. Functional integrity of labeled components was determined both by hemolytic assays and, with the exception of C'5, by participation in the generation of biological activity in the free solution systems to be described.

Immunological Reagents and Procedures

Antisera.—Rabbit and horse antisera to whole human serum were obtained commercially (Behringwerke, Marburg-Lahr, West Germany). Antisera to human C'3, C'4, and C'5 were raised in rabbits by Dr. J. T. Boyer, using highly purified components prepared in this laboratory.

Immunoelectrophoresis.—Immunoelectrophoretic analyses in 1% agar were performed by the micromethod of Scheidegger (12) at a potential gradient of 9 v per cm for 70 min.

Double Diffusion in Agar.—A microprocedure using glass microscope slides covered with a gel of 1% agar was employed. Wells were punched with a commercial template (LKB-Produkter, Stockholm, Sweden). This technique, described by Crowle (13), is more sensitive than immunoelectrophoresis and was used for immunological evaluation of purity of complement components.

Chemicals and Chemical Methods

Triethanolamine-Buffered Saline (TBS).—This buffer, at pH 7.4 and ionic strength 0.15, was used as diluent in all hemolytic assays. For reactions preceding the C'3 step of immune hemolysis, it contained 1.5×10^{-4} M Ca^{2+} and 5×10^{-4} M Mg^{2+}; for subsequent reactions, it contained 8×10^{-3} M Na_2HEDTA.

Phosphate-Buffered Saline (PBS).—This buffer, at pH 7.4 and ionic strength 0.15, was used as diluent in all free solution experiments to be described. It was composed of 0.135 μ sodium chloride, 0.015 μ sodium phosphate, and 5×10^{-4}M Mg^{2+}.

Glycine-Buffered Saline (GBS).—This buffer, at pH 3.5 and ionic strength 0.15, was used, unless otherwise indicated, for equilibration and elution of gel filtration columns and as diluent in sucrose density gradient ultracentrifugation. It was composed of 0.149 μ sodium chloride and 0.001 μ glycine buffer at pH 3.5.

Protein Determinations.—Protein concentrations of components of complement were either measured by the method of Lowry and coworkers (14) or estimated by absorption at 280 mμ, assuming a value for $E^{1\%}_{1\,cm}$ of 10. For estimation of minute amounts of protein or polypeptide in retarded fractions from gel filtration columns and in concentrated preparations of such fractions, absorption at 210 mμ was employed, assuming a value for $E^{1\%}_{1\,cm}$ of 205.

Gel Filtration.—Polyacrilamide gels (Bio-Gel P-60, 50–100 mesh; Bio-Gel P-10, 50–150 mesh) were obtained from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-75 (40–120 μ) was obtained from Pharmacia, Uppsala, Sweden. Glass columns of 1.5 cm diameter were packed under gravity flow in equilibration buffer to heights of 30 to 80 cm in various experiments. Elution was accomplished at 4°C at flow rates of 5–30 ml per hr. Fractions of equal volumes were collected by means of a volumetric siphon or drop counter. The elution positions of substances with known molecular weights were determined in the same manner. For this purpose, Blue Dextran 2000 (Pharmacia) was used to define the V_0 (void volume) of the column. Cytochrome *c* (Nutritional Biochemicals Corp., Cleveland, Ohio), porcine zinc insulin and glucagon hydrochloride (Eli Lilly and Co., Indianapolis, Ind.) were selected as molecular weight markers of 12,400, 5800, and 3485, respectively. All of these were used at a concentration of 1% and were dissolved in GBS. The elution profile of Blue Dextran was monitored by reading absorption of fractions at 600 mμ; cytochrome *c* at 410 mμ; and insulin and glucagon at 280 mμ. The position of elution of ^{125}I-anaphylatoxin was compared with these markers and its molecular weight estimated by the method of Andrews (15).

Sucrose Density Gradient Ultracentrifugation.—The technique of Martin and Ames (16), modified as described previously (17), was used to produce linear 5–20% sucrose gradients in a volume of 4.5 ml. Samples (0.2 ml) were layered on the gradients and centrifuged in a swinging bucket rotor (SW-39) in the model L Spinco ultracentrifuge (Beckman Instruments, Inc.,

Spinco Div., Palo Alto, Calif.) at 35,000 rpm for 20 hr at a chamber temperature of 5–10°C. Each lusteroid centrifuge tube was then emptied by piercing the bottom with a fine gauge needle and collecting 19–21 fractions of 18 drops each.

For purposes of monitoring the efficiency of protein separation, the sample of one of three gradients used in each run consisted of 0.2 ml of a mixture of 1% human γG-globulin (American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y.) and 1% cytochrome *c*. The fractions obtained from this tube were diluted with 3.0 ml of 0.15 M NaCl and read at 210 and 410 mμ, respectively, to define the positions of molecular weight markers of 144,000 and 12,400.

Measurement of Radioactivity.—A scintillation well counter (Model 2804, Picker Nuclear Instruments, Cleveland, Ohio) was used to measure the radioactivity of ^{125}I-labeled components of complement and fractions obtained from reaction mixtures containing a labeled component. Samples were placed in 12 × 75 mm polystyrene tubes in uniform volumes, inserted into carrier tubes, and counted for 1.0 min.

Concentration of Isolated Anaphylatoxin.—Biologically active fractions retarded on gel filtration columns were pooled and concentrated by ultrafiltration at 4°C at pressures of 30-40 psi of N_2 in a Diaplex Ultrafil apparatus, Model 50 fitted with Diaflo UM-2 or UM-3 membranes (Amicon Corporation, Cambridge, Mass.). The concentrated material in GBS buffer (pH 3.5) was used at once or frozen at −60°C. Neutralization to pH 7.4, where necessary, was performed immediately before use.

Proteolytic Enzymes.—The susceptibility of isolated anaphylatoxin to destruction by endo- and exopeptidases was tested during incubation at 37°C at indicated concentrations and pH values. The enzymes employed were: trypsin (2 × crystallized), α-chymotrypsin (3 × crystallized), and carboxy-peptidase B, all obtained from Worthington Biochemical Corp., Freehold, N. J.

Biological Materials and Procedures

Histamine, Bradykinin, Triprolidine, Pontamine Sky Blue, and Rat Agar Anaphylatoxin.—These materials were obtained and used exactly as described in a previous paper (1).

Contraction of Smooth Muscle Preparations, Enhancement of Vascular Permeability, Degranulation of Mast Cells in Guinea Pig Mesentery, and Release of Histamine from Isolated Rat Peritoneal Mast Cells.—These procedures were performed exactly as described in a previous paper (1) by modifications of published methods (4, 18–21).

RESULTS

Characterization of the Smooth Muscle–Contracting Activity Generated from Mixtures of Purified Human C′1 Esterase, C′4, C′2, and C′3 as a Cleavage Product of C′3

Preliminary Experiments and General Experimental Design.—Initial attempts to isolate biologically active fractions from reaction mixtures of C′1 esterase, C′4, C′2, and C′3 by gel filtration or sucrose density gradient ultracentrifugation were unsuccessful. In these experiments, in which fractionation was performed in PBS buffer at pH 7.4, smooth muscle–contracting activity could usually not be detected in any individual fraction or any combination of fractions. It was further observed that most of the activity in the original reaction mixture was no longer present after standing at 4°C for periods of time corresponding to the duration of gel filtration or ultracentrifuge runs. After considerable trial and error, it was found that smooth muscle–contracting activity,

once generated, could be stabilized by acidification to pH values of 2–5, representing an independent confirmation (5) of a previous report by Stegemann, Vogt, and Friedberg (22). This empiric observation, later confirmed by Müller-Eberhard and coworkers (23), made possible the following experimental approach.

Individual purified components of human complement were labeled with ^{125}I, were shown to retain functional activity, and were then employed in reaction mixtures with other unlabeled components. The labeled component was either used as is or diluted with the same unlabeled component with qualitatively similar results. Reaction mixtures consisted of C'1 esterase (5 units per ml), C'4 (50 μg per ml), C'2 (50–75 μg per ml), and C'3 (500 μg per ml) in PBS buffer (pH 7.4 phosphate-buffered saline, 5×10^{-4} M Mg^{2+}). Three of the components of complement were preincubated at 37°C for 3 min. The missing, prewarmed component (C'1 esterase) was added at zero time. Incubation was continued at 37°C for 10 min, an aliquot (0.05 ml) was removed and put into ice for immunoelectrophoresis (IEP), and the remainder of the solution was then brought to pH 3.5 with 0.1 N HCl and cooled in ice. The sample for IEP was not acidified because of loss of reactivity of C'3 with antiserum at pH 3.5. A control mixture was always prepared of identical composition to the experimental, except that one component of complement was omitted and replaced by PBS buffer. In one experiment to be illustrated, ^{125}I-C'5 (26 μg per ml) was included in the reaction mixture, all other conditions remaining the same. In this case, the C'5 was used entirely in the form of labeled component.

Experimental and control mixtures were assayed for contraction of guinea pig ileum and immunoelectrophoretic conversion of C'3. In all cases, the control was inactive on ileum and C'3 was unaltered in IEP; the complete mixture contracted guinea pig ileum with tachyphylaxis at a test dose of 0.05 ml in a 5 ml organ bath and showed complete conversion of C'3 to anodically faster components in IEP. The IEP patterns obtained are represented by Fig. 3 in reference (1) and will not be repeated here.

Acidified experimental and control mixtures (0.2 ml) were then applied to 5–20% sucrose density gradients in GBS buffer (pH 3.5 glycine-buffered saline). The experimental mixture was applied to a column (1.5 × 70–80 cm) of Biogel P-60 in GBS buffer (2.8 ml of sample), the control being held over night at 4°C for application to the same P-60 column at the time of completion of the experimental run. Finally, a mixture of Blue Dextran and cytochrome *c* was placed on the same column as markers for the V_0 of the column and the position of elution of a protein of molecular weight 12,400.

Gel filtration fractions (3.0 ml) were assayed for contraction of guinea pig ileum at a test dose of 0.5 ml and sucrose density gradient fractions (*circa* 0.2 ml) at a dose of 0.1 ml. Because of tachyphylaxis and the variability of different segments of ileum, no attempt was made to quantify the relative smooth

muscle–contracting activity of various fractions and results are recorded only as presence or absence of detectable activity. The region of biological activity of retarded fractions from P-60 columns was usually signaled by a small increase in absorption at 210 mμ. Radioactivity was measured in aliquots (1.0 ml) of the gel filtration fractions and in the entire fraction from sucrose density gradients prior to testing on ileum. Results are expressed graphically, plotting the position of fractions from P-60 columns and sucrose density gradients vs. radioactivity and biological activity. Positions of markers are also included. For purposes of graphic presentation, the relatively small amount of labeled fragment obtained in some experiments has been magnified by uniform use of a logarithmic scale for plotting radioactivity (ordinate).

Experiments with ^{125}I*-C′3.*—A typical experiment with ^{125}I-C′3 and unlabeled C′1 esterase, C′4, and C′2 in sucrose density gradient ultracentrifugation is shown in Fig. 1 (left). It is apparent that this reaction mixture, which contained biological activity, yielded a low molecular weight, labeled product associated with smooth muscle–contracting activity. A control mixture of identical composition except for absence of C′1 esterase was biologically inactive and failed to yield a low molecular weight, labeled product. This result, coupled with immunoelectrophoretic conversion of C′3 only in the complete reaction mixture, was highly suggestive of a mechanism involving cleavage of C′3 to yield a biologically active fragment.

Comparable data were obtained when the same mixtures were applied to columns of Biogel P-60 (Fig. 1, right). Retarded fractions forming a radioactivity peak associated with ileum-contracting activity were obtained from the reaction mixture of all four components. This finding was somewhat compromised by the presence of a very highly retarded radioactivity peak in the control mixture, the exact nature of which is unknown. However, this peak was present in both the complete and control mixtures and was not related to biological activity, while the earlier peak associated with ileum contraction was seen only in the complete mixture. The conclusion appeared reasonable, therefore, that fractions eluting at 83–126 ml from Biogel P-60 corresponded to fractions sedimenting at 3.9–4.3 ml in sucrose gradients.

The amount of radioactivity present in low molecular weight fractions obtained by these two techniques was about 5% of the total radioactivity recovered. The active fragment behaved as a molecule of lower sedimentation velocity and smaller hydrodynamic volume than cytochrome *c*. The sedimentation velocity of C′3 was not detectably altered, further indicating the small size of the active fragment, and also providing evidence for a discrete cleavage into large and small pieces of the C′3 molecule, rather than into multiple fragments. The significance of the difference in amount of very rapidly sedimenting material in the complete and control mixtures in Fig. 1 (left) is not known.

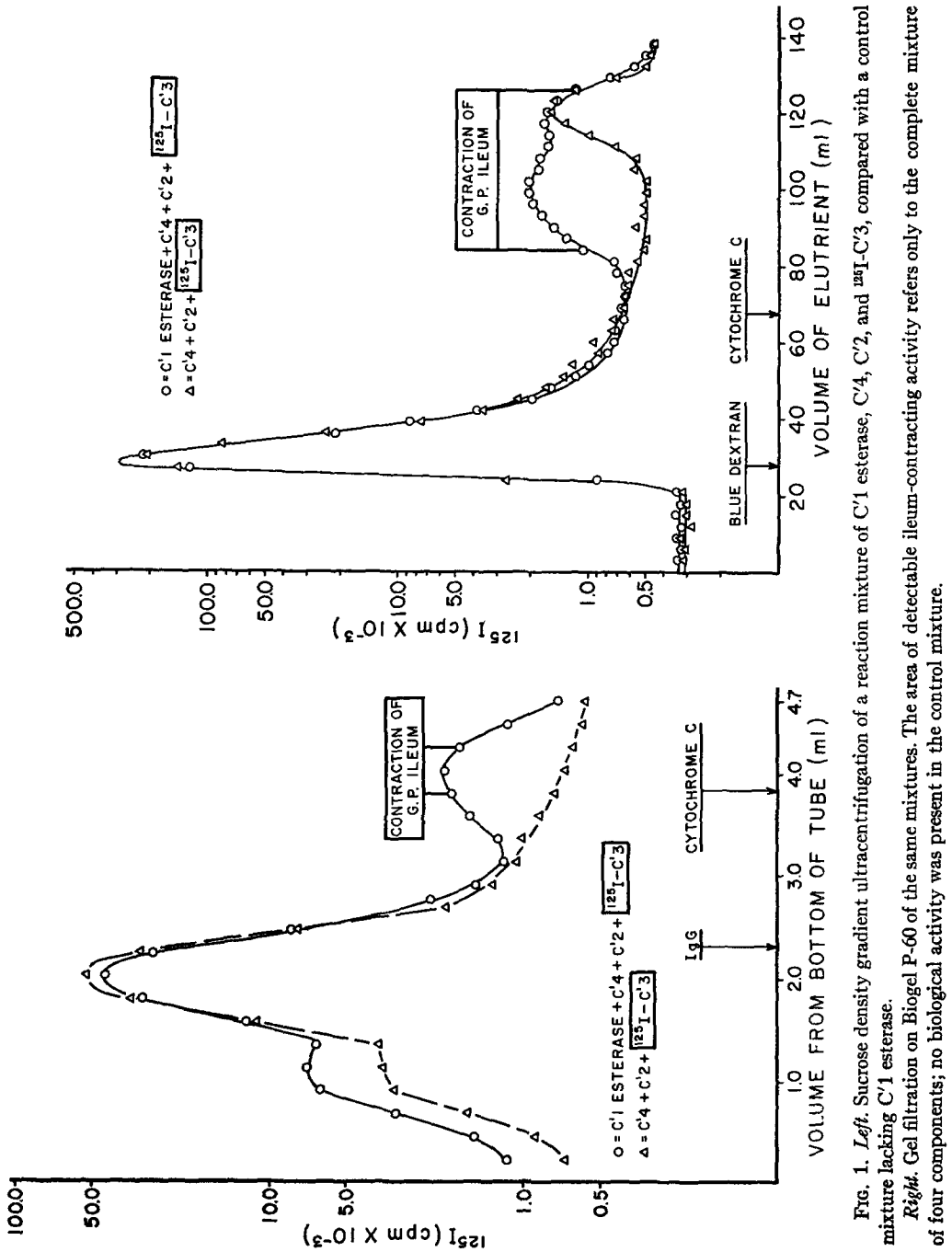

FIG. 1. *Left.* Sucrose density gradient ultracentrifugation of a reaction mixture of C'1 esterase, C'4, C'2, and ^{125}I-C'3, compared with a control mixture lacking C'1 esterase.

Right. Gel filtration on Biogel P-60 of the same mixtures. The area of detectable ileum-contracting activity refers only to the complete mixture of four components; no biological activity was present in the control mixture.

Experiments with ^{125}I-C'1 Esterase, ^{125}I-C'4, and ^{125}I-C'2.—The data presented in the previous section strongly indicated that generation of smooth muscle–contracting activity in mixtures of C'1 esterase, C'4, C'2, and C'3 was a consequence of cleavage of C'3. However, before this conclusion could be drawn with greater certainty, it was necessary to perform similar experiments with other labeled components. In each case, ileum-contracting activity was always found in low molecular weight fractions obtained from sucrose gradients or columns of Biogel P-60, but these fractions were not associated with a radioactivity peak. Thus, only when C'3 was labeled did increased radioactivity appear in the fractions having biological activity. Several interesting ancillary observations also emerged from this series of experiments.

When ^{125}I-C'1 esterase was employed with unlabeled C'4, C'2, and C'3, (C'2 being omitted in the control mixture), it was clear that ileum-contracting activity was not associated with a radioactivity peak in either sucrose density gradient ultracentrifugation or gel filtration (Fig. 2). Of separate interest was the distribution of radioactivity of C'1 esterase into lighter and heavier peaks in sucrose density gradient ultracentrifugation (Fig. 2, left). Since a highly purified preparation of C'1 esterase had been labeled, it was expected that only the lighter peak (*circa* 4S) would be found. Indeed, when the same preparation of ^{125}I-C'1 esterase was subjected to sucrose density gradient ultracentrifugation at pH 3.5 in the absence of other components, a single radioactivity peak in the 4S position was obtained. The heavier peak, therefore, most probably represented a complex between C'1 esterase and another constituent of the reaction mixture. Since the same dual peaks were observed in the control lacking C'2 (Fig. 2, left), this component could be ruled out. The relative importance of C'4 and C'3 in this phenomenon has not yet been determined, although the propensity of C'4 to complex with other proteins has been described (24).

When ^{125}I-C'4 was employed with unlabeled C'1 esterase, C'2, and C'3, (C'1 esterase being omitted in the control mixture), ileum-contracting activity was not associated with a radioactivity peak in separations performed by gel filtration (Fig. 3, right). The distribution of radioactivity of C'4 in sucrose density gradient ultracentrifugation (Fig. 3, left) indicated the presence of a low molecular weight fragment of C'4 in the complete mixture of four components. Ileum-contracting activity was found along the descending portion of this radioactivity peak, rather than with the peak itself. A fragment of C'4 had been unsuccessfully sought in previous experiments on the effect of C'1 esterase on C'4, in which a slight reduction in sedimentation velocity of the main peak of C'4 seen in Fig. 3 (left) was also observed (24). The possibility that the slowly sedimenting peak of radioactivity in Fig. 3 is indeed a fragment of C'4 which is visualized at pH 3.5 but not at pH 7 (24), requires further exploration.

When ^{125}I-C'2 was employed with unlabeled C'1 esterase, C'4, and C'3, (C'4

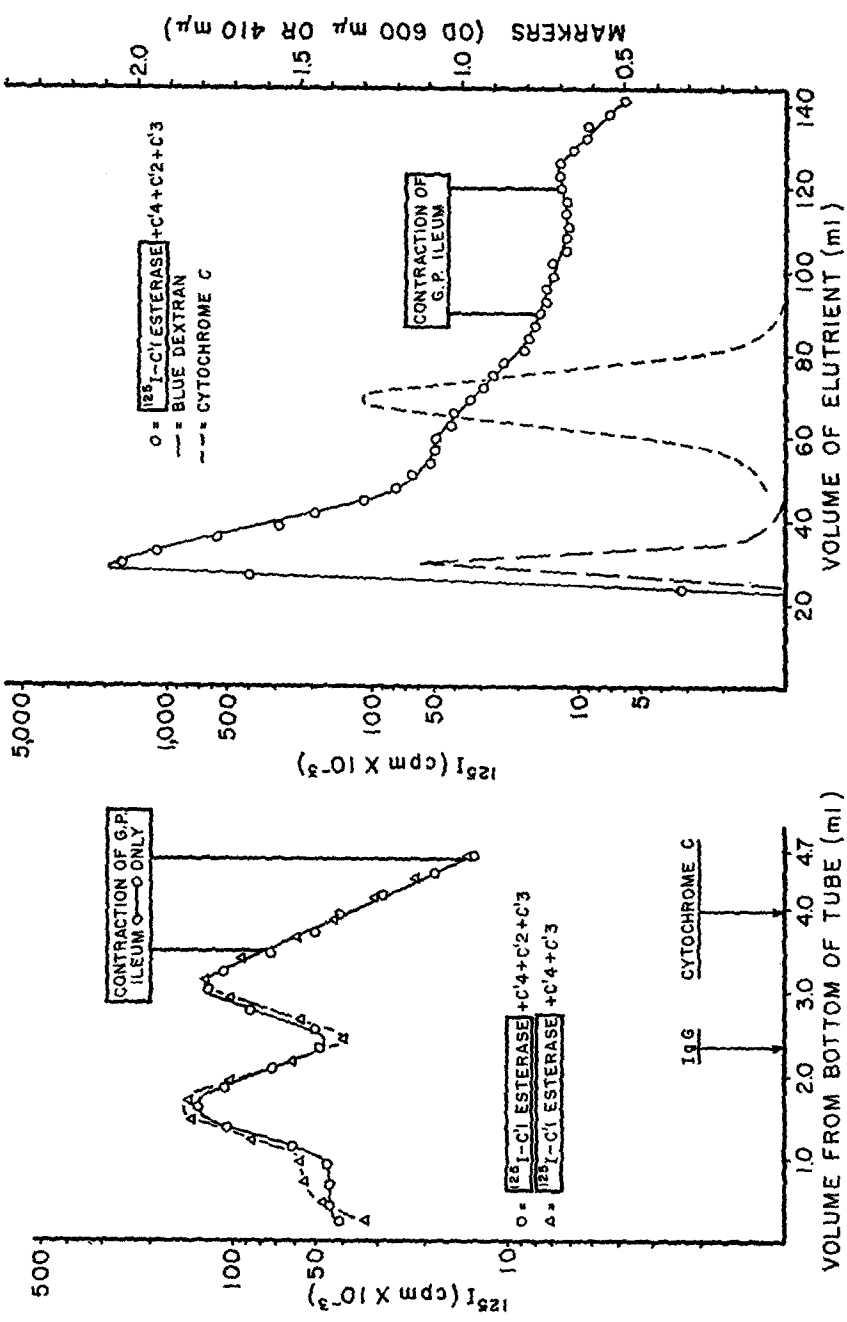

FIG. 2. *Left.* Sucrose density gradient ultracentrifugation of a reaction mixture of ^{125}I-C'1 esterase, C'4, C'2, and C'3, compared with a control mixture lacking C'2.

Right. Gel filtration on Biogel P-60 of the same mixture of four components; the control mixture gave essentially identical results and is omitted for purposes of clarity.

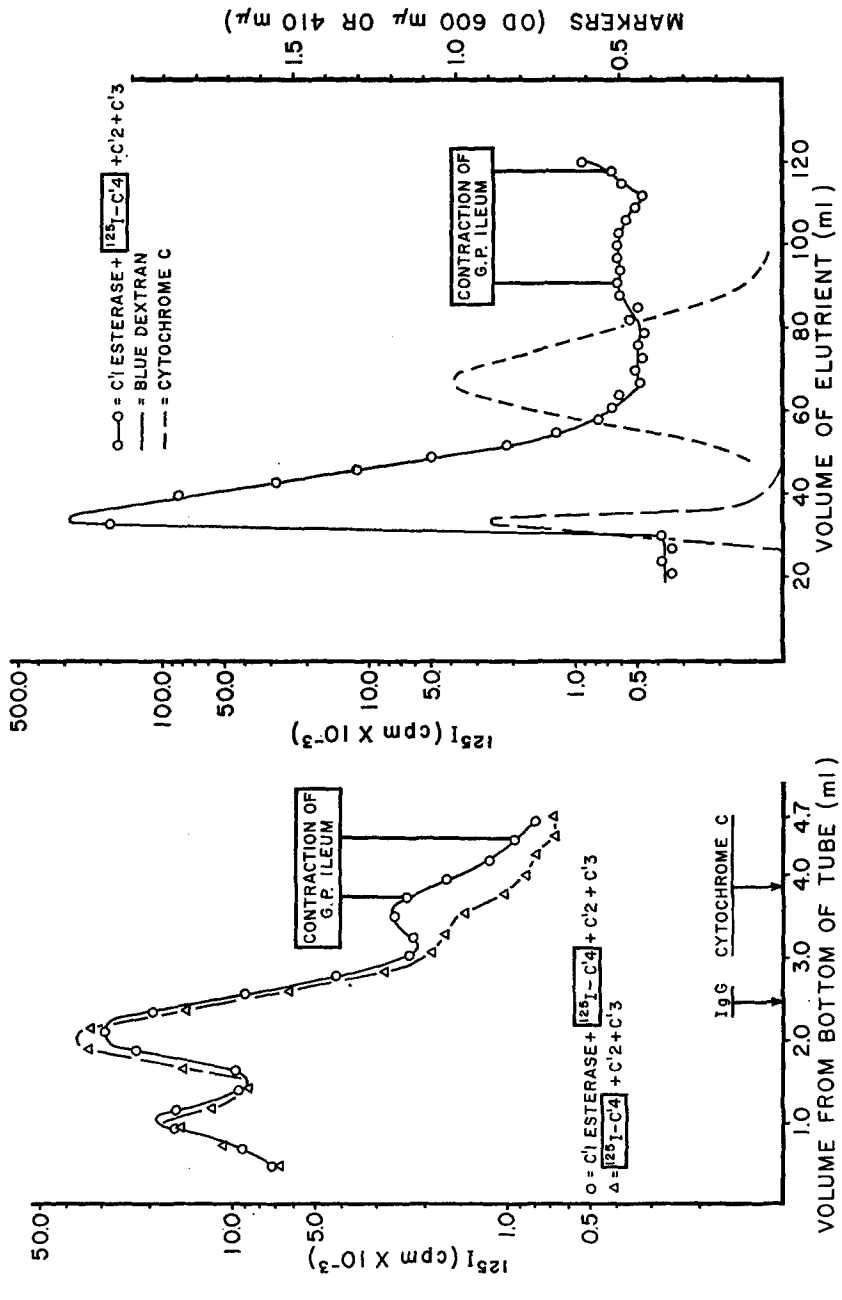

FIG. 3. *Left.* Sucrose density gradient ultracentrifugation of a reaction mixture of C'1 esterase, ^{125}I-C'4, C'2, and C'3, compared with a control mixture lacking C'1 esterase.

Right. Gel filtration on Biogel P-60 of the same mixture of four components; the control mixture gave essentially identical results and is omitted for purposes of clarity.

being omitted in the control mixture), it was again apparent that ileum-contracting activity was not associated with a radioactivity peak (Fig. 4). In this case, the distribution of radioactivity of C'2 in sucrose density gradient ultracentrifugation was essentially the same in the complete mixture and the control.

In summary, labeling of C'1 esterase, C'4, or C'2 indicated that none of these components served as a direct source of the biologically active fragment generated from mixtures of C'1 esterase, C'4, C'2, and C'3. Although all four of these components were required in the reaction mixture, the active fragment appeared to be derived only from C'3.

Experiments with ^{125}I-C'5.—It was previously reported that addition of C'5 (11–113 μg per ml) to mixtures of C'1 esterase, C'4, C'2, and C'3 neither augmented nor inhibited the generation of smooth muscle contracting activity (1). The possibility nevertheless existed that cleavage of C'5 might have occurred without affecting measurable biological activity. Accordingly, experiments were performed with ^{125}I-C'5 and unlabeled C'1 esterase, C'4, C'2, and C'3. A control lacking C'1 esterase but containing the other four components was also prepared. The results (Fig. 5) failed to provide any evidence of C'5 cleavage under the conditions of the experiment. Not shown are an additional control lacking C'3 but containing C'1 esterase, C'4, C'2, and ^{125}I-C'5, and another reaction mixture of five components containing 30 μg per ml of a different batch of ^{125}I-C'5. These yielded results entirely comparable to those shown in Fig. 5. These experiments, therefore, provided further confirmation of the conclusion that the smooth muscle–contracting activity generated in these systems resided in a cleavage product of C'3.

Experiments with ^{125}I-C'3 at pH 7.4.—In all of the experiments described above, the reaction was performed at pH 7.4 but the mixture was then brought to pH 3.5 and separations carried out at pH 3.5. When mixtures of C'1 esterase, C'4, C'2, and ^{125}I-C'3 were prepared at pH 7.4, and sucrose density gradient ultracentrifugation and gel filtration performed at pH 7.4, low molecular weight fractions with detectable ileum-contracting activity were not obtained, despite the presence of activity in the freshly prepared mixtures. Furthermore, the well-defined radioactivity peaks near the top of gradients or in a retarded position in gel filtration, as in Fig. 1 at pH 3.5, were replaced by shallow elevations of radioactivity only slightly greater than baseline levels. These findings were in accordance with preliminary failures to isolate biologically active fractions from reaction mixtures at pH 7.4, and provided additional evidence for the identity of the labeled fragment in Fig. 1 with the activity responsible for ileum contraction. The observations also lend support to the possibility that the fragment undergoes an aggregration or association reaction at pH 7.4 which is prevented or reversed at low pH, as proposed by Müller-Eberhard and coworkers (23).

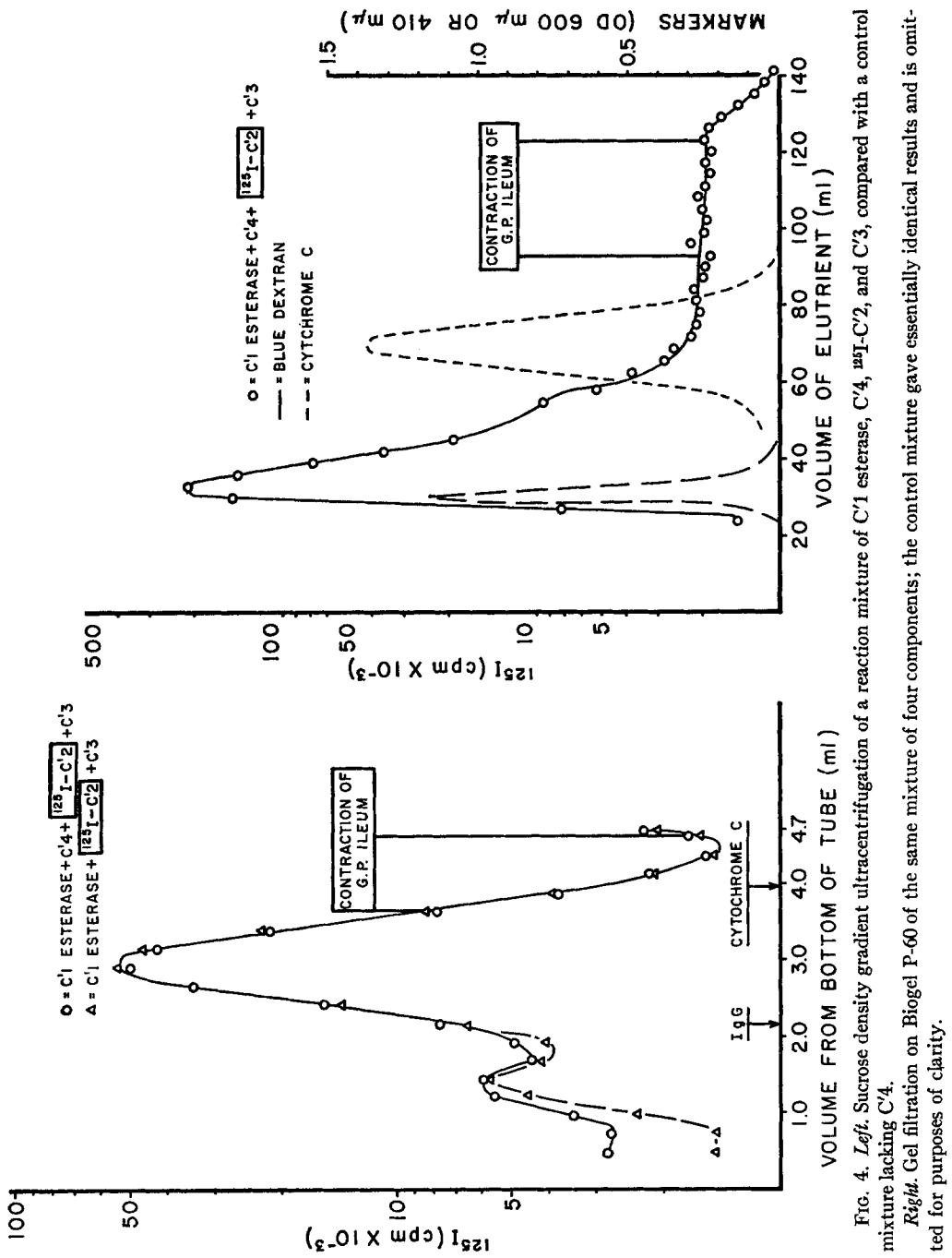

FIG. 4. *Left.* Sucrose density gradient ultracentrifugation of a reaction mixture of C'1 esterase, C'4, ^{125}I-C'2, and C'3, compared with a control mixture lacking C'4.

Right. Gel filtration on Biogel P-60 of the same mixture of four components; the control mixture gave essentially identical results and is omitted for purposes of clarity.

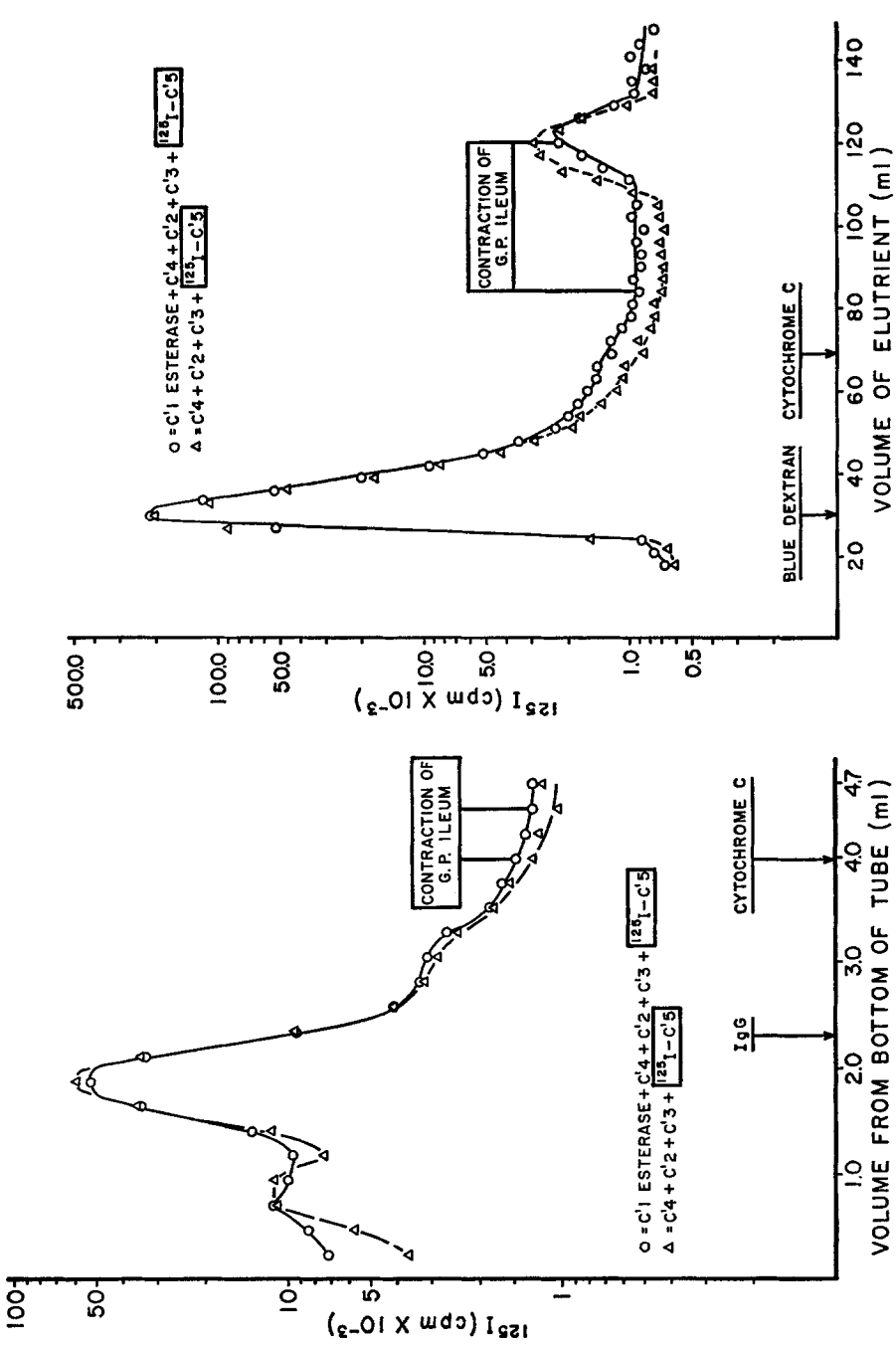

FIG. 5. *Left.* Sucrose density gradient ultracentrifugation of a reaction mixture of C'1 esterase, C'4, C'2, C'3, and ^{125}I-C'5, compared with a control mixture lacking C'1 esterase.

Right. Gel filtration on Biogel P-60 of the same mixtures. The area of detectable ileum-contracting activity refers only to the complete mixture of five components; no biological activity was present in the control mixture.

Additional Parameters Affecting the Generation of Smooth Muscle–Contracting Activity from Mixtures of Purified C'1 Esterase, C'4, C'2, and C'3

It was shown previously that generation of biological activity from mixtures of C'1 esterase, C'4, C'2, and C'3 occurred very rapidly at 37°C within physiological concentrations of these reactants, required Mg^{2+}, and was associated with immunoelectrophoretic conversion of C'3 (1). A few additional parameters have now been studied from the standpoint of the probability of an enzymatic mediation of C'3 cleavage. The concentrations of components employed in this series of experiments were 5 units per ml of C'1 esterase, 50 μg per ml of C'4, 50 μg per ml of C'2, and 500 μg per ml of C'3.

Effect of pH.—C'1 esterase, C'4, C'2, and C'3 were dialyzed against 0.15 M NaCl containing 5×10^{-4} M Mg^{2+}. Michaelis universal buffers (acetate-barbiturate) at 0.15 μ were prepared over a wide range of pH. Reaction mixtures were constructed in such a way that 37% of the total volumes consisted of Michaelis buffers. Final pH values, after incubation at 37°C for 5 min, varied in increments between 3.5 and 8.8. Activity on guinea pig ileum was assayed at a test dose of 0.05 ml (pH 3.5 and 5.0) or 0.02 ml (all other pH values). The results (Table I) demonstrated an optimum in the region of physiological pH for generation of ileum-contracting activity, with marked inhibition at pH 6.0 and 8.8. More precise definition of the pH optimum was precluded by the variability of the guinea pig ileum assay.

TABLE I

Effect of pH on Generation of Smooth Muscle–Contracting Activity in Mixtures of C'1 Esterase, C'4, C'2, and C'3

Final pH	3.5	5.0	6.0	7.0	7.4	7.9	8.5	8.8
Contraction of guinea pig ileum, *mm*	0	0	8	90	80	76	62	12

Effect of Preincubating Mixtures of C'1 Esterase and C'4 Prior to Addition of C'2 and C'3.—C'1 esterase and C'4 were incubated together in PBS buffer at 37°C for periods between 0 and 60 min, at which time the mixtures were completed by addition of C'2 and C'3 in PBS buffer. After further incubation at 37°C for 5 min, the samples were cooled in ice and assayed on guinea pig ileum at a test dose of 0.05 ml. No difference in activity of any of these samples was detectable. All elicited a full contraction on first application and a partial contraction on second application to ileum. Thus, interaction of C'1 esterase and C'4 did not yield a product subject to decay as a function of time.

Effect of Preincubating Mixtures of C'1 Esterase, C'4, and C'2 Prior to Addition of C'3.—C'1 esterase, C'4, and C'2 were incubated together in PBS buffer at 37°C for 0, 2, 5, 10, 20, 30, and 45 min, at which time the mixtures were com-

pleted by addition of C'3 in PBS buffer. After further incubation at 37°C for 5 min, the samples were cooled in ice, assayed on guinea pig ileum at a test dose of 0.05 ml, and analyzed for immunoelectrophoretic (IEP) conversion of C'3. The results (Table II) indicated rapid decay of the mixture of C'1 esterase, C'4, and C'2, and a satisfactory correlation between generation of ileum-contracting activity and IEP conversion of C'3. In separate experiments, it was shown that the decay was not attributable to instability of any of the reactants individually. Indeed, preincubation of mixtures of C'4, C'2, and C'3 at 37°C for periods of time up to 60 min did not adversely affect generation of ileum-contracting activity upon addition of C'1 esterase. Since it had already

TABLE II

Effect of Preincubation of C'1 Esterase, C'4, and C'2

Preincubation at 37°C for various periods of time prior to addition of C'3 on generation of smooth-muscle contracting activity.

Time at 37°C	Contraction of guinea pig ileum*	IEP conversion of C'3‡
min		
0	4+	4+
2	4+	4+
5	2+	4+
10	±	2+
20	0	0
30	0	0
45	0	0

* Expressed on a scale of 0 to 4+, where 4+ represents a full contraction.

‡ Expressed on a scale of 0 to 4+, where 4+ represents complete IEP conversion of C'3 to anodically faster components.

been shown that mixtures of C'1 esterase and C'4 were not subject to decay, it was concluded that further interaction with C'2 yielded a labile reactant.

All available data on the biochemical characteristics of the interaction of C'1 esterase, C'4, C'2, and C'3, leading to generation of biological activity, are fully consistent with an enzymatic mechanism in which the penultimate step is activation of C'2 and the final event is cleavage of C'3. Although our experiments were not directed to this point, they are consistent with the report of Müller-Eberhard and coworkers (23) that the action of C'1 esterase on C'4 and C'2 leads to formation of an enzyme, C'4-C'2a ("C'3 convertase"), which then acts on C'3.

General Properites of the Isolated Fragment of C'3-F(a)C'3

Nomenclature.—It will be shown in a later section that the fragment of C'3 with ileum-contracting activity, demonstrated in Fig. 1, possesses all of the

biological properties previously described for mixtures of C'1 esterase, C'4, C'2, and C'3 (1). The isolated fragment, according to existing criteria, may therefore be designated as anaphylatoxin. However, it has been found in separate studies that a fragment of C'5 may also be obtained which has similar as well as unique biological properties and which also qualifies for designation as anaphylatoxin[2] (25). Accordingly, the term anaphylatoxin is biologically descriptive of more than one chemical substance of different substrate origins and a more definitive nomenclature is now required. In an informally constituted group of active investigators in complement research, it has been agreed to pattern the designation of fragments and polypeptide chains of complement components, as they are obtained, after the system adopted for immunoglobulins. The cleavage product described in this paper is therefore designated F(a)C'3, indicating that it is a fragment, F, of a complement component, C'3. Use of the symbol (a) is arbitrary and may be interpreted either as the first fragment found or as an abbreviation of anaphylatoxin. The letter is enclosed in parentheses in an attempt to avoid possible confusion with other usage of the letter *a* for an activated state of a complement component. The remaining large fragment of the C'3 molecule would then be indicated as F(b)C'3. The cleavage reaction mediated by prior acting components of complement may therefore by written:

$$C'3 \xrightarrow{C'4 - C'2a} F(a)C'3 + F(b)C'3$$

Preparative Isolation and Concentration of F(a)C'3.—For preparative purposes, reaction mixtures of C'1 esterase, C'4, C'2, and C'3 in PBS buffer were constructed in 5–8 ml volumes, incubated at 37°C for 10 min, and acidified to pH 3.5. Concentrations of reactants were either maintained as in previous experiments or proportionately increased up to 50% to generate larger amounts of F(a)C'3. The mixture was shown to be active on guinea pig ileum and an unacidified sample was analyzed immunoelectrophoretically for complete conversion of C'3. The acidified mixture was applied to a column (1.5 × 80 cm) of G-75 Sephadex equilibrated with GBS buffer (pH 3.5) and eluted with GBS buffer in 3.0 ml fractions at a flow rate of about 20 ml per hr. G-75 Sephadex has been found to give somewhat sharper resolution and better recoveries than Biogel P-60. The emergence of retarded fractions was indicated by a shallow peak of absorption at 210 mμ, maximal values reaching an OD of about 0.25. The limits of the zone of biological activity were defined by assay on guinea pig ileum at a test dose of 0.5 ml. These corresponded to the more central portion of the OD peak. Active fractions were pooled (about 30 ml)

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[2] Dias da Silva, W., J. W. Eisele, and I. H. Lepow. Complement as a mediator of inflammation. IV. Isolation and biological properties of trypsin-split products of human C'3 and C'5. Manuscript in preparation.

and concentrated to about 5 ml in Diaplex Ultrafil apparatus with Diaflo UM-2 or UM-3 membranes. The final concentration of F(a)C'3 was estimated at about 30 μg per ml by absorption at 210 mμ.

Stability of F(a)C'3.—Concentrated preparations of F(a)C'3, maintained in GBS buffer, retained activity on guinea pig ileum for at least 24 hr at 4°C or 1 month at −70°C. Samples to be stored at low temperature were shell-frozen rapidly in dry ice–acetone. No detectable loss of activity on guinea pig ileum occurred when F(a)C'3 was heated at 80°C for 30 min in GBS buffer; partial inactivation occurred at 100°C for 30 min. Significant inactivation was observed after heating at 80°C for 30 min at pH 7.2 but detectable activity was still present. It was apparent that isolated F(a)C'3 was quite stable to spontaneous or thermal inactivation under these conditions.

Estimated Molecular Weight of F(a)C'3.—Employing cytochrome *c*, insulin monomer, and glucagon as molecular weight markers on columns of Biogel P-10 in GBS buffer (pH 3.5), attempts were made to estimate the molecular weight of F(a)C'3 by the method of Andrews (15). For this purpose, reaction mixtures of C'1 esterase, C'4, C'2, and ^{125}I-C'3 were applied to a calibrated column of P-10 and the volume of emergence (V_e) of the peak of the retarded radioactivity zone was determined. The position of this point on a standard curve, relating the values of V_e for the markers as a function of the logarithm of their molecular weights, was used to estimate the molecular weight of labeled F(a)C'3. It was recognized that the results obtained would be influenced by additional parameters, such as asymmetry and hydration. In practice, heterogeneous radioactivity peaks were obtained with variably discrete resolution into several species of decreasing molecular weight. The highest molecular weight observed was 6800. Although the suggestion was present that the lower molecular weight species might represent various dissociation products of the heaviest component, data now available do not permit this interpretation as a firm conclusion.

Susceptibility of F(a)C'3 to Destruction by Endo- and Exopeptidases.—Aliquots of a concentrated preparation of F(a)C'3 were incubated at 37°C for 10 min with 3.3 μg per ml of trypsin, α-chymotrypsin, or carboxypeptidase B at pH 7.4 in PBS buffer. The samples were cooled in ice and tested on guinea pig ileum. Treatment with each of these enzymes under these conditions resulted in complete loss of biological activity. These results were taken as supporting evidence that F(a)C'3 was, at least in part, polypeptide in nature.

Biological Properties of F(a)C'3

Concentrated preparations of isolated F(a)C'3 were tested by previously described procedures for various biological properties known to be present in the original mixture of C'1 esterase, C'4, C'2, and C'3 from which the fragment was generated (1). The results, summarized in Table III, demonstrated that

all of the biological properties of the original mixture were associated with F(a)C'3. Higher molecular weight fractions eluting with the V_0 of Biogel P-60 or Sephadex G-75 columns were biologically inactive. It was therefore concluded that the low molecular weight cleavage product of C'3, designated F(a)C'3, was itself the anaphylatoxin activity generated by interaction of the first four components of human complement in free solution.

TABLE III

Summary of Biological Properties of Isolated F(a)C'3

Biological property*	Effect of F(a)C'3‡
Contraction of guinea pig ileum	Highly active (0.01 ml): tachyphylaxis; inhibited by triprolidine
Cross-densensitization of guinea pig ileum with rat agar anaphylatoxin	No cross-desensitization: contracted ileum previously desensitized with rat agar anaphylatoxin and vice versa
Contraction of rat uterus	Inactive (0.1 ml)
Enhancement of vascular permeability in guinea pig skin	Moderately active (0.1 ml): 4.3 mm of blueing, compared with 1.0 mm for buffer control, and 8.3 mm for 0.5 μg histamine; inhibited by triprolidine
Degranulation of mast cells in guinea pig mesentery preparations	Active (0.9 ml): 55% reduction of metachromatically staining mast cells with morphological alterations of some residual cells
Histamine release from suspensions of rat peritoneal mast cells	Active: linear dose-response curve; 30% release with 0.8 ml

* For methodology, see reference (1).

‡ Preparations of isolated, concentrated F(a)C'3 were estimated to contain about 30 μg per ml.

DISCUSSION

Experiments presented in this paper have demonstrated that the action of C'1 esterase on C'4 and C'2 in the presence of Mg^{2+} produces an activity capable of cleaving C'3 into large and small fragments. Essentially the same conclusions have been reached by Müller-Eberhard and coworkers (23) and Cochrane and Müller-Eberhard (26), in turn based in part on studies in this laboratory (1, 5). The small fragment, now designated F(a)C'3 in a nomenclature system consistent with that employed for immunoglobulin degradation products, was shown to possess all of the biological properties previously found in unfractionated reaction mixtures of C'1 esterase, C'4, C'2, and C'3 (1). F(a)C'3 could therefore be identified as the anaphylatoxin generated in this free solution system of purified components of human complement. The isolated fragment had an estimated molecular weight of 6800 or less by gel filtration techniques at pH 3.5, was thermostable, and was susceptible to inactivation by trypsin,

α-chymotrypsin, and carboxypeptidase B. F(a)C'3 therefore behaved as a large polypeptide or small protein fragment. The larger product of the reaction apparently represented the remainder of the C'3 molecule and was designated F(b)C'3. Isolation of pure F(b)C'3 from reaction mixtures is in progress, preparatory to amino acid and end-group analyses of C'3 and each of these products.

Although it is now established that F(a)C'3 is an anaphylatoxin generated by the action of prior reacting components of complement on C'3, the question remains of the inability to detect this property in fresh human serum incubated with C'1 esterase or agar (4). Since we have found in unpublished experiments that both of these agents cause immunoelectrophoretic (IEP) conversion of C'3, the hypothesis is reasonable that F(a)C'3 may be generated in whole serum but that one or more mechanisms may exist by which it is rapidly bound or inactivated. Indeed, it has been our experience that mixtures of crude fractions of complement components cannot be employed for generation of anaphylatoxin, despite IEP conversion of C'3. This again suggests the existence of mechanisms for blocking the activity of a fragment which may well have been formed. Investigations on the existence and nature of such mechanisms have not yet been systematically performed.

The ability of F(a)C'3 to liberate histamine from rat peritoneal mast cells stands in contradistinction to the lack of activity in this system of classical anaphylatoxin, generated from fresh rat serum by agar (27). It is also noteworthy that F(a)C'3 contracted guinea pig ileum previously desensitized by repetitive additions of rat agar anaphylatoxin, and vice versa. Thus, although F(a)C'3 shares many of the biological properties of classical anaphylatoxin, it also behaves in part in a unique fashion. In recent experiments we have found that the unique properties of human F(a)C'3 are not attributable to inherent differences between rat and human anaphylatoxins but rather to the existence of another fragment of different substrate origin[2] (25). Treatment of human C'5 with 2 μg per ml of trypsin at 37°C for 5 min liberates a fragment which contracts guinea pig ileum with tachyphylaxis,[3] fails to contract rat uterus, enhances vascular permeability in guinea pig skin, and degranulates mast cells in guinea pig mesentery preparations. However, this fragment fails to release histamine from rat peritoneal mast cells and does not contract guinea pig ileum previously desensitized with rat agar anaphylatoxin. Conversely, if ileum is desensitized with the C'5 fragment, it will not respond to a dilution of rat agar anaphylatoxin but will contract upon addition of F(a)C'3. The trypsin-split product of C'5 further differs from F(a)C'3 in molecular weight, a value closer to that of cytochrome *c* being obtained by gel filtration at pH

[3] Contraction of guinea pig ileum with tachyphylaxis by mixtures of human C'5 and trypsin was observed first by Drs. C. G. Cochrane and H. J. Müller-Eberhard in unpublished experiments.

3.5. It would appear, therefore, that this fragment of C'5 corresponds to classical anaphylatoxin and to the activity generated by Jensen (28) from guinea pig C'3b(C'5). On the basis of cross-desensitization of guinea pig ileum, it would also be predicted to correspond to the anaphylatoxin formed by incubation of fresh guinea pig or rat serum with C'1 esterase (4). In these terms, F(a)C'3 is a "new" anaphylatoxin, the possible existence of which is only sparsely and indirectly intimated in the literature (29). Anaphylatoxin is, accordingly, a term descriptive of a general range of biological properties which are in part shared by distinctly different protein fragments derived from different components of complement. The possibility is also admissable at the present time that a fragment with similar properties may be obtained by mechanisms completely independent of a component of complement (30). Although these considerations introduce further complexities into an already complex area, they also provide a basis of understanding of apparently conflicting data (1, 28) and a further impetus for investigations on the role of complement in the pathogenesis of the acute inflammatory response.

SUMMARY

Purified preparations of human C'1 esterase, C'4, C'2, C'3, and C'5 were labeled with ^{125}I. Reaction mixtures were prepared containing a single labeled component and other unlabled components. After incubation at 37°C for 10 min at pH 7.4 in the presence of 5×10^{-4} M Mg^{2+}, they were adjusted to pH 3.5 and subjected to sucrose density gradient ultracentrifugation and gel filtration at pH 3.5. In all cases, an activity capable of contracting guinea pig ileum with tachyphylaxis was obtained in low molecular weight fractions. However, these fractions were labeled only when ^{125}I-C'3 was employed, indicating that biological activity was associated with a cleavage product of C'3. This fragment has been designated F(a)C'3 in a nomenclature consistent with that of immunoglobulin degradation products. The much larger, residual portion of the C'3 molecule has been designated F(b)C'3.

The biochemical characteristics of generation of F(a)C'3 were consistent with a mechanism involving action of C'1 esterase on C'4 and C'2, activation of C'2, and cleavage of C'3.

F(a)C'3 had a molecular weight by gel filtration techniques of 6800 or less. It was thermostable and susceptible to inactivation by endo- and exopeptidases. The isolated fragment possessed all of the biological properties of unfractionated mixtures of C'1 esterase, C'4, C'2, and C'3. In addition to contraction of guinea pig ileum, these included failure to contract rat uterus, enhancement of vascular permeability in guinea pig skin, degranulation of mast cells in guinea pig mesentery, and release of histamine from rat peritoneal mast cells. F(a)C'3 did not cross-desensitize guinea pig ileum to rat agar anaphylatoxin and vice versa.

The existence of different protein fragments with anaphylatoxin properties has been discussed. Distinctive characteristics of F(a)C'3 from classical anaphylatoxin generated by treatment of fresh rat serum with agar have been indicated.

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BIBLIOGRAPHY

1. Dias da Silva, W., and I. H. Lepow. 1967. Complement as a mediator of inflammation. II. Biological properties of anaphylatoxin prepared with purified components of human complement. *J. Exptl. Med.* **125**:921.
2. Ratnoff, O. D., and I. H. Lepow. 1963. Complement as a mediator of inflammation. Enhancement of vascular permeability by purified human C'1 esterase. *J. Exptl. Med.* **118**:681.
3. Smink, R. D., Jr., R. W. Abernethy, O. D. Ratnoff, and I. H. Lepow. 1964. Enhancement of vascular permeability by mixtures of C'1 esterase and normal serum. *Proc. Soc. Exptl. Biol. Med.* **116**:280.
4. Dias da Silva, W., and I. H. Lepow. 1965. Anaphylatoxin formation by purified human C'1 esterase. *J. Immunol.* **95**:1080.
5. Dias da Silva, W., and I. H. Lepow. 1966. Properties of anaphylatoxin prepared from purified components of human complement. Abstracts of the Complement Workshop. *Immunochemistry.* **3**:497.
6. Haines, A. L., and I. H. Lepow. 1964. Studies on human C'1 esterase. I. Purification and enzymatic properties. *J. Immunol.* **92**:456.
7. Müller-Eberhard, H. J., and C. E. Biro. 1963. Isolation and description of the fourth component of human complement. *J. Exptl. Med.* **118**:447.
8. Lepow, I. H., E. W. Todd, R. D. Smink, Jr., and J. Pensky. 1965. Purification of the second component (C'2) of human complement. *Federation Proc.* **24**:446. (Abstr.)
9. Nilsson, U. R., and H. J. Müller-Eberhard. 1965. Isolation of β_{1F}-globulin from human serum and its characterization as the fifth component of complement. *J. Exptl. Med.* **122**:277.
10. Dalmasso, A. P., and H. J. Müller-Eberhard. 1966. Hemolytic activity of lipoprotein-depleted serum and the effect of certain anions on complement. *J. Immunol.* **97**:680.
11. Helmkamp, R. W., R. L. Goodland, W. F. Bale, I. L. Spar, and L. E. Mutschler. 1960. High specific activity iodination of γ-globulins with iodine-I^{131} monochloride. *Cancer Res.* **20**:1495.
12. Scheidegger, J. J. 1955. Une micro-méthode l'immuno-électrophorèse. *Intern. Arch. Allergy Appl. Immunol.* **33**:11.
13. Crowle, A. J. 1961. Immunodiffusion techniques. *In* Immunodiffusion. Academic Press Inc., New York. 181.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.

15. Andrews, P. 1965. The gel filtration behavior of proteins related to their molecular weights over a wide range. *Biochem. J.* **98**:595.
16. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**:1372.
17. Naff, G. B., J. Pensky, and I. H. Lepow. 1964. The macromolecular nature of the first component of human complement. *J. Exptl. Med.* **119**:593.
18. Miles, A. A., and D. L. Wilhelm. 1955. Enzyme-like globulins from serum reproducing the vascular phenomena of inflammation. I. An activable permeability factor and its inhibitor in guinea pig serum. *Brit. J. Exptl. Path.* **36**:71.
19. Mota, I., and W. Dias da Silva. 1960. The anti-anaphylactic and histamine-releasing properties of the antihistamines. *Brit. J. Pharmacol.* **15**:396.
20. Dias da Silva, W., and A. D. L. Fernandes. 1965. Study of the mechanism of inhibition produced by hexoses on histamine release activity of dextran. *Experientia.* **21**:96.
21. Feldberg, W., and J. Talesnik. 1953. Reduction of tissue histamine by compound 48-80. *J. Physiol. (London).* **120**:550.
22. Stegemann, H., W. Vogt, and K. D. Friedberg. 1964. Über die Natur des Anaphylatoxins. *Hoppe-Seylers Z. Physiol. Chem.* **337**:269.
23. Müller-Eberhard, H. J., M. J. Polley, and M. A. Calcott. 1967. Formation and functional significance of a molecular complex derived from the second and fourth component of human complement. *J. Exptl. Med.* **125**:359.
24. Müller-Eberhard, H. J., and I. H. Lepow. 1965. C'1 esterase effect on activity and physicochemical properties of the fourth component of complement. *J. Exptl. Med.* **121**:819.
25. Lepow, I. H., W. Dias da Silva, and J. W. Eisele. 1968. Nature and biological properties of human anaphylatoxin. *In* The Biochemistry of the Acute Allergic Reactions. K. F. Austen and E. L. Becker, editors. Blackwell Scientific Publications Ltd., Oxford. In press.
26. Cochrane, C. G., and H. J. Müller-Eberhard. 1967. Biological effects of C'3 fragmentation. *Federation Proc.* **26**:362. (Abstr.)
27. Mota, I. 1957. Action of anaphylactic shock and anaphylatoxin on mast cells and histamine in rats. *Brit. J. Pharmacol.* **12**:453.
28. Jensen, J. 1967. Anaphylatoxin in its relation to the complement system. *Science.* **155**:1122.
29. Archer, G. T. 1960. Release of histamine from mast cells by antigen-antibody reactions in vitro. *Australian J. Exptl. Biol. Med. Sci.* **38**:147.
30. Vogt, W., and G. Schmidt. 1966. Formation of anaphylatoxin in rat plasma, a specific enzymic process. *Biochem. Pharmacol.* **15**:905.