# COMPLEMENT AS A MEDIATOR OF INFLAMMATION

## II. BIOLOGICAL PROPERTIES OF ANAPHYLATOXIN PREPARED WITH PURIFIED COMPONENTS OF HUMAN COMPLEMENT*

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In a recent paper (1) we demonstrated that interaction of highly purified human C′1 esterase with rat or guinea pig serum results in generation of an activity identical to anaphylatoxin by available criteria. The formation of this activity by C′1 esterase required the presence of serum constituents with the same heat lability as complement, was blocked by chelation of $Mg^{2+}$, was partially inhibited by salicylaldoxime or phlorizin, and was associated with essentially complete depletion of hemolytic complement activity. On the basis of these and related earlier studies (2, 3), we postulated (1) that C′1 esterase initiates formation of anaphylatoxin through reactions involving at least C′4, C′2, and C′3, possibly emerging as a reaction product at or shortly beyond the C′3 step.[1]

We have submitted this hypothesis to direct investigation, examining reaction mixtures containing various purified components of human complement for the presence of anaphylatoxin activity. It is our purpose in this paper to report successful generation of anaphylatoxin by interaction in free solution of purified C′1 esterase, C′4, C′2, and C′3. The properties of such mixtures in contracting and desensitizing smooth muscle, enhancing vascular permeability, degranulating mast cells in guinea pig mesentery preparations, and liberating histamine from isolated rat peritoneal mast cells will be described. The mechanism of formation of anaphylatoxin and its separation from complement components will be reported separately. A preliminary communication describing some of our observations has appeared previously (4).

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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.

## Materials and Methods

### Preparation and Assay 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.*—This enzyme, derived from the first component of complement, was prepared and assayed according to Haines and Lepow (5). Because of the presence of immunologically detectable C'5 in some preparations and the known migration of C'1 esterase as an $\alpha$-globulin (5), the final product was subjected to preparative electrophoresis in a Pevikon block (6). The separation was carried out at 2°C in barbital buffer, pH 8.6, $\mu = 0.05$ for 18 hr employing a potential gradient of 2.5 v per cm. The resulting product had a specific activity of 440 units per optical density unit at 280 m$\mu$, as assayed with $N$-acetyl-L-tyrosine ethyl ester as substrate, and was free of detectable C'3, C'4, and C'5 in micro–double diffusion tests in agar employing antisera specific for these purified components. It was stored at −60°C at a concentration of 100 units per ml in phosphate-buffered saline, pH 7.4.

*C'4.*—Preparations of C'4 were either kindly donated by Dr. H. J. Müller-Eberhard (designated M-E) or prepared in this laboratory (designated WRU) by modifications of the method of Müller-Eberhard and Biro (7). The well-known variability and empirical behavior of different commercial batches of TEAE- or DEAE-cellulose necessitated the changes made in the procedure. Best results were obtained with columns of DEAE-cellulose, 1.05 meq per g, type 20. A pseudoglobulin fraction of serum, prepared as described by Müller-Eberhard and Biro, was applied at equilibration conditions of pH 7.3 sodium phosphate, $\mu = 0.04$. The column was then washed exhaustively with pH 7.3 phosphate buffer, $\mu = 0.04$, containing 0.13 M NaCl (starting buffer). C'4 was eluted with a linear NaCl gradient, beginning with starting buffer and reaching a limit of 1.5 M NaCl. C'4 activity, assayed essentially as reported by Müller-Eberhard and Biro, emerged with the main protein peak at about $\mu = 0.5$ NaCl. Nearly all of the original hemolytic activity of C'4 was contained in about 200 mg protein per 100 ml of serum. This material was concentrated by ultrafiltration to 15–20 mg of protein per ml and dialyzed against second cycle starting buffer: pH 7.9 sodium-potassium phosphate, conductance at 23°C of 7 mmho/cm. It was then applied to a similarly equilibrated column of hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.), employing about 30 g of hydroxylapatite for 200 mg of protein. After exhaustive washing with equilibration buffer, step-wise elution was performed with pH 7.9 sodium-potassium phosphate buffers of conductance 11 and 13 mmho/cm. C'4 activity emerged at 13 mmho/cm without a detectable protein peak, as measured by absorption at 280 m$\mu$, in a volume of 700–900 ml. After concentration by ultrafiltration, further separation was achieved by preparative electrophoresis in a Pevikon block (6, 7). The final material contained 3–4 mg of protein per 100 ml of original serum and at least 25% of the original hemolytic activity of C'4. In micro–double diffusion tests or immunoelectrophoresis in agar, a single line was obtained with antisera to whole human serum or to purified C'4. C'3 or C'5 could not be detected with specific antisera to these components. Although most of the protein migrated as a dense band of $\beta$-globulin in disc electrophoresis, slightly faster and slower protein species were observed. The preparation was either used fresh or stored at −60°C in 50% glycerol.

*C'2.*—This protein was prepared by modifications of a method previously reported in preliminary form from this laboratory (8). Unusual difficulties have been encountered in obtaining adequate quantities of pure, hemolytically active C'2 and highly enriched preparations have therefore been used in the present study. These were purified in the range of 500- to 2000-fold with respect to serum and demonstrated good hemolytic activity, as assayed by

conversion of the intermediate complex EAC'1, 4 to EAC'1, 4, 2 according to procedures devised by Leon and described elsewhere (9). C'3, C'4, and C'5 were not detectable by micro-double diffusion tests in agar but several bands were observed in disc electrophoresis. Fractions were either used fresh or stored at −60°C either in the presence or absence of 50% glycerol.

*C'3.*—Preparations of C'3 were either kindly donated by Dr. H. J. Müller-Eberhard (designated M-E) or prepared in this laboratory (designated WRU) by a convenient modification of the method of Nilsson and Müller-Eberhard (10). In the fractionation of C'1q, C'1r, and C'1s, a protein peak highly enriched in C'3 emerges from the column of DEAE-cellulose after C'1q and before C'1r (11). Employment of this fraction for further chromatography on hydroxylapatite (10) makes possible simultaneous preparation of four factors of the complement system. Furthermore, by using a shallower gradient than originally described (11), substituting a limit buffer containing 0.35 M rather than 0.5 M NaCl, significantly less contamination with $\beta_{1H}$-globulin (10) has been observed from DEAE-cellulose chromatography. Indeed, immunoelectrophoretically pure $\beta_{1H}$-globulin appears in the chromatogram as a small peak immediately preceding the larger peak containing C'3. Commercial hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) has proven highly effective for final separation. By these means, 10–15 mg of C'3 per 100 ml of serum may be routinely obtained as a single protein species in disc electrophoresis and immunoelectrophoresis or micro-double diffusion in agar using antisera to whole human serum. No observable conversion of C'3 to immunoelectrophoretically faster forms of this protein (12) occurred during preparation. C'4 and C'5 could not be detected in micro-double diffusion tests with antisera to these purified components. The final product was either used fresh or stored at −60°C in 50% glycerol (13).

*C'5.*—A single preparation of highly purified C'5 (10) was kindly donated by Dr. H. J. Müller-Eberhard.

## Immunological Reagents and Procedures

*Antisera.*—Rabbit and horse antisera to whole human serum were obtained commercially (Behringewerke, Marburg-Lahr, West Germany). Rabbit antisera to C'3 and C'4 were raised in rabbits by Dr. J. Boyer, using highly purified components originally donated by Dr. H. J. Müller-Eberhard or prepared in this laboratory. Rabbit antiserum to C'5 (10) was supplied by Dr. H. J. Müller-Eberhard.

*Immunoelectrophoresis.*—Immunoelectrophoretic analyses were performed by the micromethod of Scheidegger (14) employing 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). The general technique has been described by Crowle (15).

## 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 (16). For reactions preceding the C'3 step of immune hemolysis, it contained $1.5 \times 10^{-4}$ M $Ca^{2+}$ and $5 \times 10^{-4}$ M $Mg^{2+}$; for subsequent reactions, it contained $8 \times 10^{-3}$ M $Na_3HEDTA$.

*Phosphate-Buffered Saline (PBS-Mg²⁺).*—This buffer, at pH 7.4 and ionic strength 0.15, was used as diluent in all biological systems to be described. It was composed of 0.135 $\mu$ sodium chloride, 0.015 $\mu$ sodium phosphate, and $5 \times 10^{-4}$ M $Mg^{2+}$.

*Salts of Ethylenediaminetetraacetic Acid (EDTA).*—Reagent grade $Na_2H_2EDTA$ was titrated to pH 7.4 at a stock concentration of 0.15 M, giving a solution largely in the form $Na_3HEDTA$. $Na_2MgEDTA$ (Geigy Chemical Corp., Ardsley, N. Y.) was titrated to pH 7.4 at a stock concentration of 0.15 M.

*Specific Conductance.*—For chromatography of C'3 and C'4, buffers for stepwise elution

from hydroxylapatite were prepared at stated values of specific conductance at 23°C (10). This was measured with a commercial conductivity meter (Radiometer Co., Copenhagen, Denmark).

*Disc Electrophoresis.*—This technique was performed with a commercial apparatus (Model 6, Canal Industrial Corp., Bethesda, Md.) according to directions supplied by the manufacturer.

*Protein Determinations.*—Protein concentrations were either measured by the method of Lowry and coworkers (17) or estimated by absorption at 280 m$\mu$, assuming a value for $E_{1cm}^{1\%}$ of 10.

## Biological Materials and Procedures

*Histamine.*—Histamine dihydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in Tyrode's solution at a stock concentration of 1 $\mu$g of histamine base per ml for contraction of isolated smooth muscle. For experiments on enhanced vascular permeability, stated concentrations in PBS-Mg$^{2+}$ buffer were used.

*Bradykinin.*—The synthetic product, kindly donated by Sandoz, Inc., Hanover, N. J., contained 80 $\mu$g per ml. It was diluted further in appropriate buffers, as for histamine.

*Triprolidine.*—Triprolidine hydrochloride (Actidil, Burroughs Wellcome and Co., Tuckahoe, N. Y.) was dissolved in Tyrode's solution at a stock concentration of 0.1 $\mu$g per ml for use as an antihistamine with smooth muscle preparations. For experiments on enhanced vascular permeability, it was incorporated with the intravenous dose of Pontamine Sky Blue such that a dose of 0.1 mg per kg was administered.

*Pontamine Sky Blue.*—Pontamine Sky Blue 6x (E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.) was dissolved in 0.075 M NaCl at a concentration of 5%.

*Contraction of Smooth Muscle Preparations.*—Assay of anaphylatoxin activity was performed routinely on isolated guinea pig ileum, as described previously (1). Segments of ileum were suspended in a 5 ml organ bath containing atropinized Tyrode's solution (10 $\mu$g atropine sulfate per liter) and maintained at 37°C with constant aeration. After establishing constant reactivity to several additions of 0.05 $\mu$g of histamine, a stated volume of test sample was added and allowed in contact for 30 sec. The preparation was then washed with fresh Tyrode's solution, retested with 0.05 $\mu$g of histamine, and washed again with fresh Tyrode's solution. Successive test samples were assayed in this manner, always allowing an interval of 1 min for equilibration of the ileum in fresh Tyrode's solution before adding either histamine or test sample. Contractions were recorded on a kymograph supplied with electrosensitive paper, employing an electrically stimulated lever arm attached to the ileum by nonconducting thread. The amplitude of contractions was measured as the perpendicular distance from the base line, uncorrected for magnification by the lever (*circa* 5x). In order to eliminate the effect of desensitization (tachyphylaxis), only one test sample was assayed with each segment of guinea pig ileum. In the few cases in which rat uterus was used for additional tests, Tyrode's solution was replaced by de Jalon's solution and the temperature was reduced to 31°C. For this purpose, either Wistar or Sprague Dawley female rats were injected subcutaneously with 10 $\mu$g of stilbesterol (Eli Lilly and Co., Indianapolis, Ind.) 36 hr prior to sacrifice. The induction of early estrus was confirmed by examination of vaginal smears.

*Enhancement of Vascular Permeability.*—This property of anaphylatoxin was tested using the technique of Miles and Wilhelm (18). Noninbred albino guinea pigs of either sex, usually weighing between 400 and 450 g, were depilated and injected intravenously with 1.2 ml per kg of body weight of a 5% solution of Pontamine Sky Blue 6x. The solutions to be tested were then injected intracutaneously into the dorsum of the guinea pig in a volume of 0.1 ml, using No. 26, ¼ inch steel hypodermic needles and plastic tuberculin syringes. 15 min after the last injection, the size of the lesion was estimated by averaging the widest diameter and the diameter perpendicular to it. The results are expressed as the average of four guinea pigs.

*Degranulation of Mast Cells in Guinea Pig Mesentery.*—Fragments of guinea pig mesentery about 1 × 1 cm were shaken at 37°C for 15 min with 0.7 ml of the solutions to be tested and 0.1 ml of 1% glucose. The tissue was transferred to fixative and then stained with 0.1% toluidine blue at acid pH, as described by Mota and Dias da Silva (19). Mast cell damage was quantified by counting the number of cells containing metachromatic granules in 20 microscopic fields (magnification of 100) and calculating the average number per field.

*Release of Histamine from Isolated Rat Peritoneal Mast Cells.*—The ability of anaphylatoxin to release histamine from suspensions of rat peritoneal cells was tested by modification of the method of Dias da Silva and Fernandes (20). Suspensions of rat peritoneal cells containing about 5% mast cells were obtained by injecting 15 ml of PBS-$Mg^{2+}$ buffer containing 5 μg per ml of heparin and 0.1% gelatin into the peritoneal cavity of 200-g Wistar or Sprague Dawley rats of either sex, which had previously been anesthetized with ether and exsanguinated by decapitation. The abdominal wall was gently massaged for 1 min and then incised along the midventral line. Fluid in the peritoneum was withdrawn with a 5 ml syringe without needle and transferred to plastic tubes at room temperature. The cells were sedimented by centrifugation for 5 min at 50 g and washed twice with the same buffer used for peritoneal injection. The cell yield from three rats was suspended in about 1 ml of the same buffer and held at room temperature for use within 30 min.

Assay mixtures were composed of 0.8 ml of test solution or PBS-$Mg^{2+}$ buffer, 0.1 ml of 1% glucose and 0.1 ml of cell suspension. After incubation at 37°C for 10 min, the cells were sedimented by centrifugation for 3 min at 400 g. The supernatant fluids were carefully decanted, 1 drop of 1% phenol red was added, and the solution acidified with 2 drops of 0.1 N HCl. The sedimented cells were resuspended in 1 ml of PBS-$Mg^{2+}$ buffer and acidified in the same manner. All samples were placed in a boiling water bath for 30 min. Neutralization with 0.1 N NaOH was performed just prior to assay.

Histamine content of each supernatant and sedimented sample was assayed on guinea pig ileum, according to Feldberg and Talesnik (21). The results were expressed as per cent of total histamine liberated into the fluid phase.

*Preparation of Rat and Guinea Pig Anaphylatoxin.*—For experiments on cross-desensitization of guinea pig ileum by anaphylatoxins derived from different species, rat and guinea pig anaphylatoxin were prepared by incubation of the corresponding plasmas with agar suspension or human C'1 esterase, as described previously (1). Plasma was obtained either by drawing blood into heparin (50 μg per ml) or Na₂MgEDTA (5 × 10⁻³ M).

## General Experimental Design

Aliquots of purified preparations of C'2, C'3, and C'4 which had been frozen were thawed and dialyzed exhaustively with stirring against PBS-$Mg^{2+}$ buffer at 2°C. C'1 esterase was thawed as needed and used without further treatment. These components usually maintained good activity in the liquid state for about 1 wk. No relevant differences were observed in the behavior of purified components which had been frozen or used shortly after preparation without prior freezing.

On the basis of preliminary trials, the following general procedure was adopted for experiments with purified components of complement. Mixtures of C'2, C'3, and C'4 and an aliquot of C'1 esterase, all at desired concentrations in PBS-$Mg^{2+}$ buffer, were preincubated separately at 37°C for 2 min. The enzyme was then added and incubation continued for an additional stated period, most frequently 5 min. The reaction mixture was chilled in an ice bath and either tested at once or acidified with N HCl or pH 3.0 glycine buffer, 0.1 M to adjust to pH 3.5–4.0. As will be reported separately, human anaphylatoxin is more stable at acid pH than in neutral solutions.

In the experiments reported here, all possible control mixtures lacking a single component were simultaneously prepared in analogous fashion. The final concentrations of the remain-

ing three reactants were identical to those in the complete system, volume being adjusted with PBS-Mg$^{2+}$ buffer.

In a few experiments, C'5 was also added to the reaction mixture. In these cases, all components other than C'1 esterase were preincubated at 37°C for 2 min prior to addition of prewarmed enzyme. The general procedure and inclusion of controls were otherwise as already described.

<div align="center">RESULTS</div>

*Generation of Anaphylatoxin from Purified Components of Human Complement and Its Activity on Smooth Muscle Preparations*

*Contraction of Guinea Pig Ileum by Reaction Mixtures Containing C'1 esterase, C'4, C'2, and C'3.*—The following is representative of a large number of experiments performed over a period of more than 1 yr with many different batches of purified components. In the cases of C'3 and C'4, preparations either donated by Dr. H. J. Müller-Eberhard (M-E) or purified in this laboratory (WRU) were employed with entirely comparable results.

A reaction mixture containing C'1 esterase, C'4, C'2, and C'3 was incubated at 37°C for 5 min and chilled in ice for immediate testing, as described under General Experimental Design. The final concentrations of reactants were: 5 units per ml of C'1 esterase, 50 μg per ml of C'4 (WRU), 50 μg per ml of C'2, and 500 μg per ml of C'3 (WRU). Controls lacking C'1 esterase, C'4, C'2, or C'3, designated 1C, 4C, 2C, and 3C respectively, were also prepared at the same final concentrations of those components present. As described in a later section, these concentrations were in considerable excess over those required to generate measurable biological activity.

As shown in Fig. 1, a single segment of guinea pig ileum, which responded well to 0.05 μg of histamine, failed to respond to additions of 0.05 ml of each control mixture which lacked one of the four components employed. However, addition of 0.05 ml of the complete reaction mixture (HA) resulted in full contraction of the ileum segment with almost no lag period. A second addition of 0.05 ml of HA produced only a small contraction after a slightly longer lag period. A third addition of 0.05 ml of HA was completely without effect, although the ileum was still fully responsive to 0.05 μg of histamine.

Desensitization of guinea pig ileum (tachyphylaxis) with successive additions of the same sample is a salient characteristic of anaphylatoxin which serves to distinguish it from many other activities capable of contracting smooth muscle. The number of applications required to achieve full desensitization and the amplitude of each contraction depend on a variety of factors, including the sensitivity of the ileum segment, the potency of the preparation, and the dose employed. The number of contractions observed with different preparation of HA has varied in our experience between one and four.

The possibility was considered that the manner of testing of samples depicted in Fig. 1 might have obscured the conceivable presence of small amounts of

activity in one or more controls and depressed the activity observed with the complete mixture. For example, the control lacking C'1 might have been unable to cause a contraction but might still have effected desensitization to a small amount of activity possibly present in the control lacking C'4. The final reactivity to the complete mixture under these circumstances might therefore also have been speciously low due to prior partial desensitization of the ileum. Accordingly, experiments with comparable mixtures were performed in which
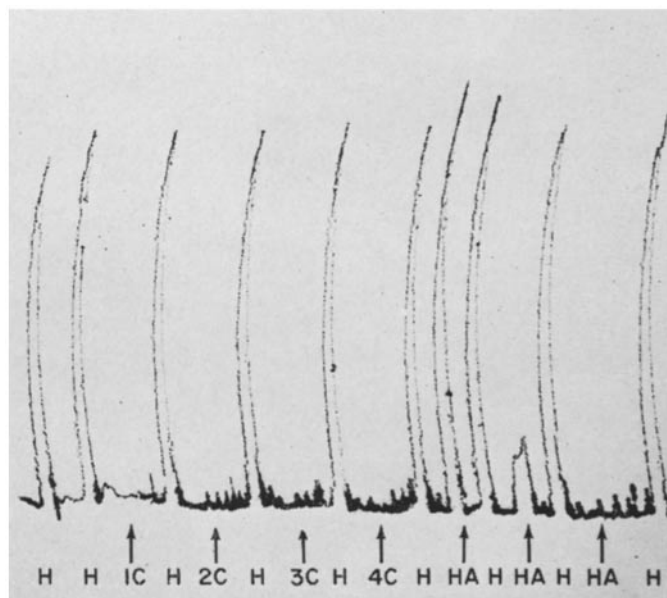


FIG. 1. Effect of mixtures of C'1 esterase, C'4, C'2, and C'3, and controls lacking one of these components, on contraction of guinea pig ileum in vitro. H = 0.05 $\mu$g histamine base; 1C, 2C, 3C, and 4C = 0.05 ml of controls lacking C'1 esterase, C'2, C'3, C'4, respectively; HA = 0.05 ml of complete mixture.

each sample was tested on a fresh segment of ileum. The absence of detectable activity in each control mixture and the unaltered activity of the complete mixture effectively ruled out the applicability of these speculations in most of the experiments reported.

These experiments clearly demonstrated that a property capable of contracting guinea pig ileum and resembling anaphylatoxin in its desensitizing characteristics could be generated from incubation mixtures of C'1 esterase, C'4, C'2, and C'3. The requirement for all four reactants was indicated by the absence of biological activity in each of the four controls lacking a single component.

*Effect of Addition of C'5 to Mixtures of C'1 esterase, C'4, C'2, and C'3.*—The effect of C'5 was tested directly by preparing reaction mixtures of C'1 esterase (2.5 units per ml), C'4 (M-E, 20 μg per ml), C'2 (50 μg per ml), and C'3 (M-E, 200 μg per ml) similar to those described in the preceding section but containing in addition 11, 22, 45, and 113 μg per ml of purified C'5. All of these samples were equally active on guinea pig ileum and could not be distinguished from a comparable mixture lacking added C'5. This conclusion was based both on the amplitude of contraction achieved with several doses of each sample and on the number of additions required to achieve desensitization at a given
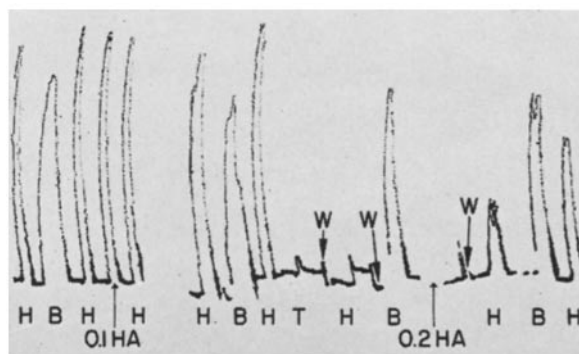


FIG. 2. Effect of triprolidine on the ability of mixtures of C'1 esterase, C'4, C'2, and C'3 (HA) to contract guinea pig ileum in vitro. H = 0.05 μg histamine base; B = 0.05 μg bradykinin; 0.1 HA and 0.2 HA = 0.1 ml and 0.2 ml of complete reaction mixture; T = 0.05 μg of triprolidine; W = wash artifact due to movement of lever during changing of Tyrode's solution in the organ bath. Two different segments of ileum are represented. On the left, the response of untreated ileum to H, B, and HA are shown. The blocking effect of triprolidine on the activity of H and HA but not of B and the gradual recovery of reactivity to H are shown on the right.

dose. It appeared therefore that purified C'5 neither augmented nor inhibited the smooth muscle–contracting activity generated from the preparations of C'1 esterase, C'4, C'2, and C'3 used in these studies.

*Effect of an Antihistamine (Triprolidine) on the Smooth Muscle–Contracting Activity Generated from Mixtures of C'1 esterase, C'4, C'2, and C'3.*—A reaction mixture containing 2 units per ml of C'1 esterase, 20 μg per ml of C'4 (M-E), 40 μg per ml of C'2, and 200 μg per ml of C'3 (M-E) was incubated at 37°C for 5 min and chilled in ice, as described previously. After establishing that 0.1 ml of the final mixture (HA) caused full contraction of a segment of guinea pig ileum, the effect of an antihistaminic drug was tested (Fig. 2).

A fresh segment of ileum from the same guinea pig was shown to respond to successive additions of 0.05 μg of histamine, bradykinin, and histamine. The

ileum was then exposed to 0.05 μg of triprolidine for 30 sec. The antihistaminic effect of this treatment was demonstrated by failure of the ileum to respond to 0.05 μg of histamine without impairment of the response to 0.05 μg of brady-kinin. Addition at this point of 0.2 ml of HA, twice the dose previously shown to be fully active, failed to cause contraction. Gradual recovery of the ileum segment was shown by increasing response to 0.05 μg of histamine at 15 and 30 min after application of triprolidine, indicating the reversibility of the anti-histaminic effect of this drug and the integrity of the ileum. As a final check on the responsiveness of the untreated ileum to HA, another segment from the same guinea pig was found to contract upon addition of 0.1 ml of HA.

In separate experiments, the possibility was excluded that the failure of triprolidine-treated ileum to respond to HA was due to inherent nonreactivity of that particular segment. For this purpose, a preparation of HA was employed which caused a strong contraction after a second application. After demonstrating nonresponsiveness of the triprolidine-treated ileum to histamine and a single dose of HA, a recovery period of about 30 min was allowed to elapse. It was found that when response to 0.05 μg of histamine reappeared, a second addition of HA then caused muscle contraction.

These experiments demonstrated that the activity generated from mixtures of C′1 esterase, C′4, C′2, and C′3 further resembled anaphylatoxin by virtue of inhibition of contraction of guinea pig ileum by an antihistamine.

*Failure of Mixtures of C′1 esterase, C′4, C′2, and C′3 to Contract Rat Uterus.* —A reaction mixture containing 5 units per ml of C′1 esterase, 50 μg per ml of C′4 (WRU), 50 μg per ml of C′2, and 500 μg per ml of C′3 (WRU) was incubated at 37°C for 5 min, chilled in ice, and brought to pH 3.7 with pH 3.0 glycine buffer. In tests on guinea pig ileum, 0.05 ml of this sample (HA) caused full contraction and three applications at this dose were required for complete desensitization. The same mixture was then tested on uterine horn from a rat in early estrus. The responsiveness of this muscle preparation to 1 ng of brady-kinin was first established and 0.1 ml of HA was then applied. No contraction was observed, although the horn was still reactive to a second dose of 1 ng of bradykinin.

*Summary of Component Requirements for Generation of Smooth Muscle-Con-tracting Activity and Its Anaphylatoxin-Like Properties.* —These experiments demonstrated that an activity capable of contracting guinea pig ileum could be generated from reaction mixtures containing C′1 esterase, C′4, C′2, and C′3 and that the presence of all four of these components was required. The further addition of C′5 was without potentiating or inhibitory effect on the system, suggesting that obligatory component requirements were met at the C′3 step. The ability of the activity generated to contract and desensitize guinea pig ileum, the blocking effect of an antihistamine, and the failure to contract rat

uterus were compatible with tentative identification of the activity as anaphylatoxin.

### Some Parameters Affecting the Generation of a Smooth Muscle-Contracting Activity from Mixtures of Purified C'1 esterase, C'4, C'2, and C'3

Before proceeding with further biological characterization of the activity described in the previous section, a few immediately pertinent parameters of the reaction mixture of purified components were investigated. The purpose of these experiments was limited to providing assurance that adequate conditions were being employed for generation of biological activity. In all of the studies in this section, the biological reference was contraction and desensitiza-

TABLE I

Approximate Concentrations of C'1 esterase, C'4, C'2, and C'3 Required for Minimal and Maximal Contraction of Guinea Pig Ileum

| Component | Concentration for ileum contraction* | |
|---|---|---|
| | Minimal | Maximal |
| C'1 esterase | 0.05 unit per ml | 1.0 unit per ml |
| C'4 | 10 µg per ml | 25 µg per ml |
| C'2 | 10 " " " | 25 " " " |
| C'3 | 60 " " " | 200 " " " |

* Various concentrations of each component were incubated with adequate or excess concentrations of the remaining three components; 0.1 ml of reaction mixture was applied to different segments of guinea pig ileum.

tion of guinea pig ileum. More extensive experiments, bearing more directly on the mechanism of the reaction, will be reported separately.

*Effect of Concentration of Reactants.*—A series of experiments was performed in which a range of concentrations of a single component were mixed with the remaining three components at concentrations which appeared to be optimal or in excess. Incubations were at 37°C for various periods of time from 5 to 30 min. The mixtures were tested on guinea pig ileum at a dose of 0.1 ml and the results recorded as the minimum concentration of the component being titrated which was required for minimal and maximal contraction under these conditions (Table I). For example, in the titration of C'2, concentrations of C'2 varying between 5 and 100 µg per ml were incubated at 37°C for 5 min with 2.5 units per ml of C'1 esterase, 25 µg per ml of C'4 (M-E), and 200 µg per ml of C'3 (M-E). The threshold concentration of C'2 required for minimal contraction of ileum was 10 µg per ml, while full contraction at the dose applied was observed with 25 µg per ml. However, higher concentrations of reactants gave mixtures of still greater potency, as evidenced by lower dose require-

ments for ileum contraction and the number of doses required to desensitize. For this reason, multiples of the concentrations shown in Table I were usually employed.

The results in Table I were interpreted only as a qualitative indication of the concentrations of particular preparations of reactants giving adequate biological activity. The difficulties of quantifying the system further are compounded not only by the possibility that different concentrations of a given reactant might influence the threshold concentration of another but also by the inherent limitations of a biological assay which requires testing on separate segments of ileum which may vary significantly in reactivity. However, these experiments provided a guide for further studies. They also gave an indication

TABLE II

*Effect of Sodium and Magnesium Salts of EDTA on the Generation of Smooth Muscle-Contracting Activity from Mixtures of C'1 Esterase, C'4, C'2, and C'3*

| Salt of EDTA | Contraction of guinea pig ileum | | IEP conversion of C'3* |
|---|---|---|---|
| | First dose | Second dose | |
| | *mm* | *mm* | |
| None‡ | 87 | 8 | 4+ |
| Na₃HEDTA | 0 | 0 | 0 |
| Na₂MgEDTA§ | 94 | 4 | 4+ |

\* Designates extent of immunoelectrophoretic conversion of C'3 from the native form which migrates cathodically to anodically faster species; 4+ refers to complete conversion.

‡ Sample tested on same segment of ileum which failed to respond to sample containing Na₃HEDTA.

§ Sample tested on fresh segment of ileum from same guinea pig.

that the required concentrations of reactants were physiologically realistic. The concentrations of C'2, C'3, and C'4 giving maximal activity in Table I were roughly comparable to normal serum concentrations which have been estimated for these components and the amount of C'1 esterase was far less than that which is potentially available in normal serum. It should be emphasized that the final concentrations of reactants in the ileum assay were actually only one-fiftieth of those shown, since 0.1 ml of sample was added to a 5 ml organ bath.

*Requirement for* $Mg^{2+}$.—The effect of chelation of divalent cations on the generation of smooth muscle–contracting activity from mixtures of purified components of complement was studied employing the sodium and magnesium salts of EDTA. Reaction mixtures consisting of 5 units per ml of C'1 esterase, 50 µg per ml of C'4 (WRU), 50 µg per ml of C'2, and 500 µg per ml of C'3 (WRU) were incubated at 37°C for 5 min in the absence of EDTA or in the presence of either 5 × 10⁻³ M Na₃HEDTA or Na₂MgEDTA. The resulting samples were tested on guinea pig ileum at a dose of 0.1 ml. As shown in Table

II, Na$_3$HEDTA completely inhibited the generation of biological activity from this mixture, while Na$_2$MgEDTA was without effect as compared with the control. The presence of Na$_3$HEDTA also blocked the immunoelectrophoretic conversion of C'3 to anodically faster components, as presented in a later section. These results, compatible with a requirement for Mg$^{2+}$ but not Ca$^{2+}$ in the reaction mechanism, provided the basis for routine incorporation of Mg$^{2+}$ into the buffer used as diluent in all reaction mixtures (PBS-Mg$^{2+}$).

*Effect of Time of Incubation at 37°C.*—In this experiment, all reactants were mixed at 0°C to give final concentrations of 5 units per ml of C'1 esterase, 50 μg per ml of C'4 (WRU), 50 μg per ml of C'2, and 500 μg per ml of C'3 (WRU). Zero time was noted as the time of addition of C'1 esterase to the remaining reactants. The mixture was placed immediately in a 37°C water bath. Samples of 0.18 ml were withdrawn into 0.02 ml of 0.05 M Na$_3$HEDTA at 0.5, 2, 5, 15, and 30 min, chilled in ice, and tested as soon as possible on guinea pig ileum at a dose of 0.05 ml. A comparable control was included in which C'1 esterase was omitted.

All samples removed from the tube containing the complete reaction mixture were fully active on guinea pig ileum and no activity was observed in any of the control samples. Thus, under the conditions employed, the generation of a smooth muscle–contracting property from C'1 esterase, C'4, C'2, and C'3 occurred within 30 sec at 37°C and was not adversely affected by incubation for 30 min. These results provided assurance of the adequacy of incubation times of 5–30 min. It was recognized that a time curve might have been demonstrated at lower concentrations of reactants and at a lower test dose on guinea pig ileum but such experiments were not germane to our purpose at this time.

*Immunoelectrophoretic Analysis (IEP) of Reaction Mixtures.*—An excellent correlation was observed between the generation of smooth muscle–contracting activity from mixtures of purified components and the conversion of C'3 to antigenically related but anodically faster migrating species in IEP. Reaction mixtures to be tested in IEP were not acidified because of alterations in the immunological behavior of C'3. Controls lacking a single component and devoid of biological activity all showed a pattern in IEP identical to that of C'3 alone. In the presence of adequate concentrations of C'1 esterase, C'4 and C'2, C'3 was fully converted to a major very fast component and a minor component in the area of the antigen well, both of which reacted with antiserum prepared against purified C'3 or whole human serum. In all such cases, the mixtures were capable of contracting guinea pig ileum.

A representative experiment is shown in Fig. 3. Although in this instance the bottom well contained a control lacking only C'1 esterase, the same results were obtained with controls lacking only C'2 or C'4 or with C'3 itself. The

top well, containing all four reactants incubated at 37°C for 5 min, appeared exactly the same when the mixture was tested after incubation for periods of 0.5–30 min, as described in the previous section. For this purpose, the agar used for IEP contained $5 \times 10^{-3}$ M Na₃HEDTA in an attempt to inhibit further conversion during electrophoresis.

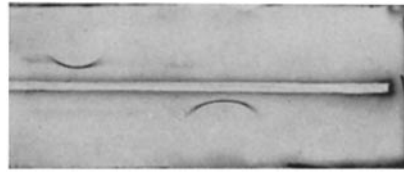On occasion and usually associated with waning activity of components, the



FIG. 3. Immunoelectrophoretic analysis of mixtures of purified components of complement incubated at 37°C for 5 min. Bottom well: C'4 (WRU, 50 μg per ml), C'2 (50 μg per ml), and C'3 (WRU, 500 μg per ml). Top well: same concentrations of reactants as in bottom well but also containing 5 units per ml of C'1 esterase. The trough contained rabbit antiserum to purified human C'3. Anode is at the left.
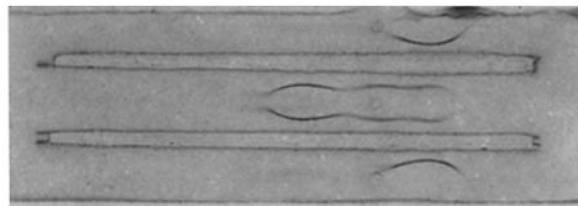


FIG. 4. Immunoelectrophoretic analysis of mixtures of purified components of complement incubated at 37°C for 5 min. Top well: C'1 esterase (2.5 units per ml), C'2 (30 μg per ml), C'3 (WRU, 200 μg per ml); middle well: same concentrations of reactants as in top well but also containing C'4 (WRU, 25 μg per ml); bottom well: C'3 alone (WRU, 200 μg per ml). The troughs contained rabbit antiserum to whole human serum. Anode is at the left. The biological activity of the complete mixture in the middle well was less than that of the corresponding mixture in Fig. 3.

pattern shown in the middle well of Fig. 4 was observed. This demonstrates residual unconverted C'3, as well as two faster protein species, all of which are antigenically closely related. In such cases, the middle arc near the antigen well was considerably more dense and distinct than shown in Fig. 3. Incomplete conversion of C'3 was associated with less smooth muscle–contracting activity than that obtained with full conversion. In separate experiments in which the concentration of C'4 was known to be limiting and only very weak biological activity was obtained, the pattern of the middle well of Fig. 4 was observed.

*Other Biological Properties of Mixtures of Purified C'1 Esterase,*
*C'4, C'2, and C'3*

*Enhancement of Vascular Permeability in Guinea Pig Skin.*—The activity of mixtures of purified C'1 esterase, C'4, C'2, and C'3 and controls lacking single components was tested for enhancement of vascular permeability in the skin of guinea pigs previously untreated or injected intravenously with an antihistaminic drug (triprolidine) (18). In view of the permeability enhancing property of higher concentrations of C'1 esterase alone (2), mixtures were made

TABLE III

*Effect of Mixtures of Purified C'1 Esterase, C'4, C'2, and C'3 and Control Mixtures Lacking Individual Components on Vascular Permeability in Guinea Pig Skin in the Presence and Absence of Triprolidine*

| Components in mixture | Permeability in guinea pig skin | |
|---|---|---|
| | Absence of triprolidine | Presence of triprolidine |
| | *mm* | *mm* |
| C'2, C'3, C'4 | 1.5 | 1.8 |
| C'1 esterase, C'4, C'3 | 5.7 | 1.4 |
| "      "    C'2, C'3 | 2.0 | 1.5 |
| "      "    C'4, C'2 | 2.3 | 1.7 |
| C'1 esterase, C'4, C'2, C'3 | | |
| (HA-Undiluted) | 5.5 | 1.6 |
| (HA-1/2) | 3.6 | 1.5 |
| (HA-1/4) | 1.8 | 1.3 |
| Histamine (50 μg per ml) | 8.5 | 1.4 |
| Bradykinin (10 μg per ml) | 6.9 | 7.5 |
| PBS-Mg$^{2+}$ | 1.1 | 1.3 |

at 37°C for 5 min with only 2 units per ml of this enzyme. C'4 (WRU) was used at a concentration of 50 μg per ml; a very highly purified preparation of C'2 was used at 10 μg per ml; and C'3 (WRU) was used at 500 μg per ml. The complete mixture (HA) was active on guinea pig ileum, three applications of 0.05 ml being required for full desensitization. When the complete mixture was diluted 1/2, two applications of 0.05 ml desensitized the ileum; when diluted 1/4, a single application gave only partial contraction. All of the controls were inactive on ileum.

Samples (0.1 ml) were then injected intradermally into two groups of four guinea pigs, one of which received only Pontamine Sky Blue intravenously while the other was given triprolidine (0.1 mg per kg) with the dye. In the

absence of triprolidine, the results (Table III) showed highly significant enhancement of vascular permeability by undiluted HA, an effect which diminished toward control values with further dilution. Controls lacking C'1, C'3, and C'4 induced permeability of vessels only slightly greater than the diluent (PBS-Mg$^{2+}$) itself. However, the control lacking C'2 was as active as the complete mixture (HA). In the presence of triprolidine, the permeability enhancing activity of HA and the control lacking C'2 was entirely inhibited.

This experiment, representative of several others, demonstrated a correlation between the ability of mixtures of C'1 esterase, C'4, C'2, and C'3 to contract guinea pig ileum and to enhance vascular permeability in guinea pig skin. There

TABLE IV

*Effect of Mixtures of Purified C'1 Esterase, C'4, C'2, and C'3 and Control Mixtures Lacking Individual Components on Reduction of Metachromatically Staining Mast Cells in Guinea Pig Mesentery Preparations*

| Components in mixture | Contraction of guinea pig ileum | Reduction of mast cells in guinea pig mesentery |
|---|---|---|
| | *mm* | *%* |
| C'2, C'3, C'4 | 0 | 0 |
| C'1 esterase, C'4, C'3 | 0 | 0 |
| "        "   C'2, C'3 | 0 | 0 |
| "        "   C'4, C'2 | 0 | 0 |
| C'1 esterase, C'4, C'2, C'3 (HA) | 80 | 33 |
| Rat plasma + agar* | 80 | 39 |

* Rat plasma containing Na$_2$Mg EDTA was incubated at 37°C for 30 min with 100 μg per ml of agar suspension.

was a further correlation in the blocking effect of triprolidine in both assay systems. An apparent discrepancy existed, however, in the lack of smooth muscle activity of mixtures of C'1 esterase, C'3, and C'4 and the presence of permeability enhancing activity in the same mixture. Since the latter property was not demonstrable in triprolidine-treated guinea pigs, it may be assumed that the activity measured, in common with anaphylatoxin, acted by way of histamine release. It has tentatively been concluded that these results are referable to the presence of guinea pig C'2 at the injection site, constituting a a complete system for generation of anaphylatoxin in vivo. The absence of activity in other controls is more readily interpretable, since mixtures lacking C'1 esterase do not have a source of active C'1 and mixtures lacking C'4 and C'3 have decayed or ineffective C'2 prior to injection. Experiments bearing on these interpretations will be presented separately.

*Effect on Mast Cells in Guinea Pig Mesentery Preparations.*—Mixtures of C'1 esterase (5 units per ml), C'4 (WRU, 50 μg per ml), C'2 (75 μg per ml), and C'3

(WRU, 500 $\mu$g per ml), incubated at 37°C for 5 min, were found to be capable of reducing the number of metachromatically staining mast cells in fragments of guinea pig mesentery. In the experiment shown in Table IV, there was an excellent correlation between the lack of activity on guinea pig ileum (0.05 ml) or mesentery (0.7 ml) of controls lacking individual components and the presence of both of these activities in the complete mixture of four components or in rat anaphylatoxin generated with agar. In addition to the recorded reduction of stainable mast cells with both of the latter preparations, there were also characteristic morphological alterations of many of the cells which stained

TABLE V

*Effect of Mixtures of Purified C'1 Esterase, C'4, C'2, and C'3 and Control Mixtures Lacking Individual Components on Release of Histamine from Isolated Rat Peritoneal Mast Cells*

| Components in mixture | Contraction of guinea pig ileum | IEP conversion of C'3* | Histamine release from rat peritoneal mast cells‡ |
|---|---|---|---|
| | *mm* | | *%* |
| C'2, C'3, C'4 | 0 | 0 | 3.3 |
| C'1 esterase, C'4, C'3 | 0 | 0 | 2.7 |
| "          "          C'2, C'3 | 5 | ± | 9.2 |
| "          "          C'4, C'2 | 0 | 0 | 3.6 |
| C'1 esterase, C'4, C'2, C'3 (HA) | 63 | 4+ | 38 |
| PBS-Mg$^{2+}$ | 0 | — | 3.8 |

* Designates extent of immunoelectrophoretic conversion of C'3 from the native form which migrates cathodically to anodically faster species; 4+ refers to complete conversion.

‡ Data for 0.8 ml of each mixture in a final reaction volume of 1.0 ml; average of experiments with two different pools of peritoneal cell suspensions each from three rats.

metachromatically with toluidine blue. These were similar to those previously described by Mota (22) for anaphylatoxin.

Although not an isolated observation, the data of Table IV are not fully representative of all experiments of this kind which have been performed. In all cases, the controls lacking individual components were inactive. However, the extent of activity observed with rat anaphylatoxin and the complete mixture of C'1 esterase, C'4, C'2, and C'3 (HA) was variable and not necessarily internally correlated. Thus, in one experiment, HA showed good activity on mesentery while rat anaphylatoxin was only marginally active; in another, HA appeared inactive and rat anaphylatoxin was highly active. The basis for this variability is not known. The empirical difficulties of this system led to direct experiments with isolated mast cells.

*Effect on Isolated Rat Peritoneal Mast Cell Preparations.*—Mixtures of C'1 esterase (5 units per ml), C'4 (WRU, 50 $\mu$g per ml), C'2 (50 $\mu$g per ml), and C'3

(WRU, 500 μg per ml), incubated at 37°C for 5 min, were highly active in releasing histamine from isolated rat peritoneal mast cell suspensions (Table V). The complete mixture (HA) also demonstrated full conversion of C'3 in immunoelectrophoretic analysis (IEP) and activity on guinea pig ileum (0.05 ml). Controls lacking C'1 esterase, C'2, and C'3 were completely inactive in releasing
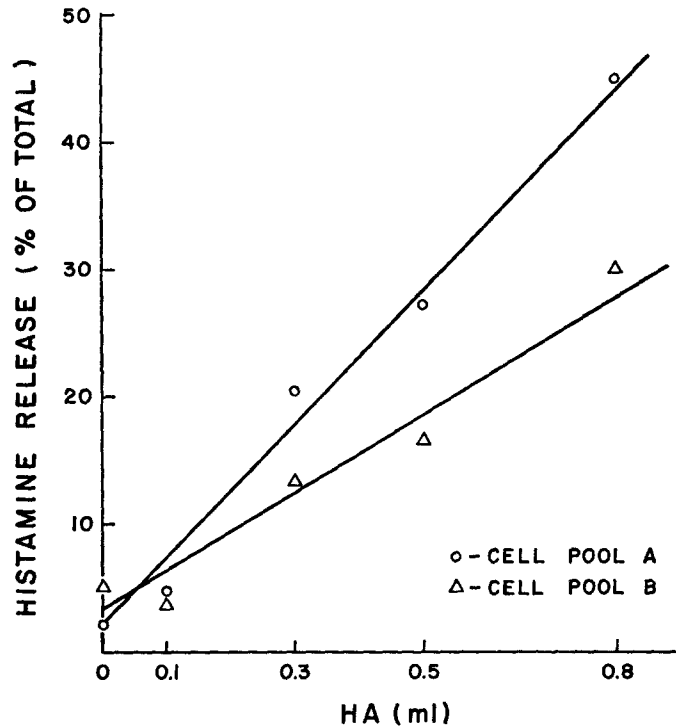


Fig. 5. Histamine release from isolated rat peritoneal mast cell suspensions by mixtures of C'1 esterase, C'4, C'2, and C'3 (HA); effect of concentration of HA on two different pools of cells.

histamine from rat peritoneal cells or contracting guinea pig ileum and showed no conversion of C'3 in IEP. However, in this experiment, the control lacking C'4 had minimal activity in the histamine and ileum assays and further showed minimally observable conversion of C'3. There was therefore an excellent correlation in these three independent systems. The explanation for the presence of detectable activities in the control lacking C'4 is not known with certainty but may be related to the presence of small amounts of C'4 or C'4-C'2 complex in this preparation of C'2, not detectable by antigenic analysis.

The effect of various concentrations of HA on histamine release from two

different pools of rat peritoneal mast cells is depicted in Fig. 5. The linearity of the dose-response curve and the quantitative variability of cell suspensions are demonstrated.

*Cross-Desensitization of Guinea Pig Ileum by Anaphylatoxins of Different Species.*—All of the experiments reported on the biological properties of the

TABLE VI

*Cross-Desensitizing Properties of Human, Rat, and Guinea Pig Anaphylatoxins Prepared by Various Procedures: Guinea Pig Ileum Assay*

| Ileum desensitized with | | Ileum challenged with | | Result of challenge |
|---|---|---|---|---|
| Species | Anaphylatoxin preparation | Species | Anaphylatoxin preparation | |
| Rat | Plasma + agar | Human | Purified components | No desensitization |
| " | "     + C'1 esterase | " | Purified components | "          " |
| Human " | Purified components | Rat | Plasma + agar | No desensitization |
| | Purified compoments | " | "     + C'1 esterase | "          " |
| Guinea pig | Plasma + agar | Human | Purified components | No desensitization |
| Human | Purified components | Guinea pig | Plasma + agar | "          " |
| Rat Guinea pig | Plasma + agar "          " | Guinea pig Rat | Plasma + agar "          " | Densitization* Partial desensitization‡ |

* Ileum failed to contract following indicated challenge but then responded to human anaphylatoxin prepared with purified components of complement.

‡ Ileum contracted following indicated challenge but was desensitized following only two applications of rat anaphylatoxin, as compared with five applications when a fresh segment of ileum was employed. Complete desensitization to both guinea pig and rat anaphylatoxins did not prevent contraction upon addition of human anaphylatoxin prepared with purified components of complement.

activity generated from mixtures of purified human C'1 esterase, C'4, C'2, and C'3 were compatible with identification of this activity as anaphylatoxin. It was therefore of interest to gain preliminary insight into the mutual identity of anaphylatoxins prepared with plasma or plasma proteins of different species, using desensitization and cross-desensitization of guinea pig ileum as a convenient criterion. For this purpose, rat anaphylatoxin was prepared by incubation of rat plasma with agar or human C'1 esterase (1); guinea pig anaphylatoxin by incubation of guinea pig plasma with agar; and human anaphylatoxin

by incubation of purified human C′1 esterase, C′4, C′2, and C′3, as described in previous sections.

In all cases, a segment of guinea pig ileum was completely desensitized by repetitive additions of anaphylatoxin from one species and then challenged with anaphylatoxin of another species. If the challenge dose elicited contraction of the previously densensitized ileum, it was concluded that anaphylatoxin of the first species did not cross-desensitize to the second species. Similarly, if the challenge dose failed to elicit a contraction, the result was interpreted as cross-desensitization between the two species. Finally, if the challenge dose elicited contraction of the previously desensitized ileum but many fewer applications of the challenge anaphylatoxin were required for subsequent desensitization, as compared with results on a fresh segment of ileum, partial cross-desensitization between the two species was indicated.

The results of this series of experiments, summarized in Table VI, showed that rat anaphylatoxin failed to cross-desensitize to human anaphylatoxin and vice versa; that guinea pig anaphylatoxin failed to cross-desensitize to human anaphylatoxin and vice versa; that rat anaphylatoxin fully desensitized to guinea pig anaphylatoxin; and that guinea pig anaphylatoxin partially desensitized to rat anaphylatoxin. It was clear from these data that anaphylatoxin generated from purified components of human complement could be differentiated on the basis of this biological parameter from rat or guinea pig anaphylatoxin but that a closer biological relationship existed between rat and guinea pig anaphylatoxins.

## DISCUSSION

Friedberger (23) introduced the term "anaphylatoxin" to designate a property that induced a syndrome similar to anaphylactic shock in guinea pigs when homologous serum was exposed to immune precipitates. A similar activity was also produced with certain polysaccharides, such as agar (24, 25), starch (26), or inulin (27). These observations were later extended to serum from other animal species, including rabbit and rat (28). A renewed interest in the mechanism of anaphylatoxin formation and the nature of anaphylatoxin itself was initiated by observations that its effects on the whole animal or on isolated smooth muscle (29) could, at least in part, be explained on the basis of its histamine-releasing properties (22, 30, 31).

Friedberger's hypothesis (32) that the complement system might be involved in anaphylatoxin formation was revived in the last decade (33, 34). In an experimental approach to this concept using immune aggregates and fresh rat serum, Osler and coworkers (35) found a direct relationship between loss in hemolytic activity of the serum, loss in activity of C′3 components, and generation of an activity which enhanced vascular permeability and contracted smooth muscle. However, more rigorous definition of the role of complement

and the nature of anaphylatoxin itself awaited purification, characterization, and elucidation of the mechanism of action of complement components.

Studies on the mechanism of anaphylatoxin formation employing purified components of complement were initiated in this laboratory by examining the effects of highly purified C'1 esterase in inducing enhancement of vascular permeability in guinea pig skin or in generating an activity indistinguishable from anaphylatoxin when incubated with rat or guinea pig serum (1–3). On the basis of these experiments, it was postulated that anaphylatoxin might be a product of interaction of C'1 esterase, C'4, C'2, C'3, and perhaps later acting components. With this information at hand and with the availability of techniques for preparing highly purified components, a direct attack on the problem became possible.

In the present report, we have indeed demonstrated that interaction of highly purified preparations of human C'1 esterase, C'4, C'2, and C'3 in the presence of $Mg^{2+}$ results in generation of an activity which, in its range of biological properties, is identifiable as anaphylatoxin. These biological effects include contraction and desensitization of guinea pig ileum, 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 rat peritoneal mast cell suspensions. The histamine-releasing property was further emphasized by the blocking effect of an antihistaminic drug, triprolidine, on contraction of guinea pig ileum and enhancement of vascular permeability.

The requirement for antigen-antibody complexes or other inducers of anaphylatoxin formation was bypassed in our experiments by constructing a free solution system in which C'1 esterase was used as the initiator of subsequent reactions of the complement sequence. The primary function of the immune complex in the complement system is to combine with C'1 in a reaction which results in creation of a catalytic center on the C'1s portion of the macromolecular complex of C'1q-C'1r-C'1s (11, 36). Although the intact activated complex of C'1 is the functional unit in immune hemolysis (36, 37), the enzymatically active subunit derived from C'1s, designated C'1 esterase, is capable of reacting in free solution with subsequently acting components. Such reactions may either be observed as inactivation of the hemolytic activity of C'4 and C'2 (37, 38) or as transient activation of a component, as in formation of the artificial intermediate complex, EC'4, by incubation of unsensitized erythrocytes with purified human C'1 esterase and C'4 (39). Thus, the use of C'1 esterase permitted examination of reaction mixtures composed entirely of solutions of complement components, uncomplicated by the presence of additional reactants or an insoluble phase.

Reaction mixtures composed of C'1 esterase, C'4, C'2, C'3, and $Mg^{2+}$ and no other detectable components of complement were adequate and sufficient

for generation of anaphylatoxin. The concentrations of these components re-
quired for evolution of measurable biological activity were within physiological
limits. Addition of pure C'5 neither enhanced nor inhibited the system, strongly
suggesting that only the first four components of complement are necessary for
anaphylatoxin formation. The present data do not permit this conclusion to be
drawn with absolute assurance since other activities, not measurable by cur-
rent methods, could conceivably have been involved. However, independent
data will be reported separately which clearly indicate that anaphylatoxin is
a relatively low molecular weight split product of C'3, confirming the present
findings that only the complement sequence as far as C'3 is necessary and as-
signing a causal relationship to immunoelectrophoretic conversion of C'3 (Figs.
3 and 4) and generation of anaphylatoxin. These experiments are based on
separation of labeled anaphylatoxin from mixtures of C'1 esterase, C'4, C'2,
and physicochemically and immunochemically pure [131]I- or ^{125}I-labeled C'3. They
are conceptually in accord with a report that rat and pig anaphylatoxins, in-
duced by the action of cobra venom on the respective plasmas, are split products
of a plasma protein (40).

An apparent discrepancy exists, however, between these findings and the ob-
servations reported by Jensen (41) from Nelson's laboratory that anaphyla-
toxin is derived from guinea pig C'3b. This conclusion is based on studies with
a purified cobra venom factor, antigen-antibody complexes, or trypsin and
"functionally purified" components of guinea pig complement. In the nomen-
clature used by Nelson for guinea pig components, C'3b appears to correspond
to human C'5. The explanation for the difference in Jensen's conclusions and
our own is not fully available at this time. Factors to be considered and in-
vestigated include possible differences of pathways with guinea pig and human
components in terms of intrinsic species variations. The possibility must also
be entertained that undetected activities or inhibitors may be present in either
Nelson's preparations of complement components or our own. For example,
preparations of pure C'4 which have lost hemolytic activity during storage at
2°C continue, for a further period, to retain the ability to participate in ana-
phylatoxin formation with C'1 esterase, C'2, C'3, and $Mg^{2+}$ in free solution.
Thus, complete reliance on hemolytic activity may not be adequate for func-
tional characterization of a purified component of complement. In addition,
since the full range of biological properties of Jensen's activity has not yet been
reported, the further possibility is admissable that C'3b may serve as a sub-
strate from which a biologically active factor may be generated which is similar
to but distinct from that described in this paper.

The ability of the activity generated from purified components of comple-
ment to liberate histamine from suspensions of rat peritoneal mast cells repre-
sents, to our knowledge, the first description of this property of anaphylatoxin.
It raised the possibility that complement-dependent, noncytotoxic histamine

release from mast cells, described by Austen and Becker (42), might be mediated by anaphylatoxin. Indeed, some of the experiments described in the present paper were being conducted at the same time as those reported by Austen and Becker and the parallelism between the two systems in the requirement for C'1, C'4, C'2, and C'3 was striking. Apparent dissociation of the two mechanisms emerged, however, when a requirement for C'5 was demonstrated in Austen and Becker's experiments but not our own. The data now available would suggest that complement-dependent, noncytotoxic histamine release from mast cells proceeds by a mechanism distinct from that operative in histamine release from mast cells by anaphylatoxin. However, direct investigation of the possible role of anaphylatoxin in the former system appears indicated.

The possible relationship of anaphylatoxin generated from mixtures of human C'1 esterase, C'4, C'2, C'3, and Mg$^{2+}$ to two other split products of C'3 which have recently been encountered must also be considered. These studies further serve to emphasize the existence of alternate pathways to the complement system and the potentially important role of products of C'3 in mediation of of events of the acute inflammatory response. Ward[2] has found that incubation of plasminogen and streptokinase with pure human C'3 results in cleavage of the C'3 molecule with liberation of a chemotactic fragment of about 6000 molecular weight. In direct collaborative experiments with Dr. Ward, we have found that the plasmin-generated product of C'3 does not contract guinea pig ileum and anaphylatoxin is not chemotactic.[3] It would appear, therefore, that plasmin and the earlier acting complement components exert a different biochemical attack on C'3 yielding split products which, at least in part, have distinct biological characteristics.

The relationship between human anaphylatoxin and the C'3 fragment recently described by Müller-Eberhard (43) has not yet been investigated. Vogt and Schmidt (44) have reported purification of a factor from cobra venom, free of proteolytic, hemolytic, and phospholipase A activities, which is capable of generating anaphylatoxin from rat serum. Müller-Eberhard and coworkers have now shown that the cobra venom factor (7S) forms a complex (9S) with a serum protein (5S) which then acts on C'3 to yield a split product with biological properties not yet fully reported but compatible with possible identity or relation to anaphylatoxin. Aside from the nature and species source of the substrate, the cobra venom system under study by Müller-Eberhard and by Jensen (41) appear entirely analogous. Direct comparison of the human and guinea pig products obtained by these investigators and the human anaphylatoxin reported here is clearly indicated.

[2] Ward, P. A. A plasmin-split product of C'3 as a new chemotactic factor. Manuscript in preparation.

[3] Ward, P. A., W. Dias da Silva, and I. H. Lepow. Unpublished data.

The experiments on cross-desensitization of guinea pig ileum by rat, guinea pig, and human anaphylatoxins were conducted initially to evaluate the biological relationships between rat–human and guinea pig–human anaphylatoxins. When no cross-desensitizing properties were observed between these pairs, it became of interest to investigate the relationship of rat and guinea pig anaphylatoxins. This was undertaken because of previous experiments quoted frequently as evidence against the participation of anaphylatoxin in organ anaphylaxis in vitro. Greisman (45) and Mota (22) showed that segments of ileum from guinea pigs sensitized in vivo to a given antigen could be made unresponsive by repetitive additions of rat anaphylatoxin in vitro but could nevertheless still contract upon addition of the specific antigen. Assuming that the addition of antigen might have initiated liberation of guinea pig anaphylatoxin, the validity of the interpretation placed on such experiments depends on the cross-desensitizing property of rat and guinea pig anaphylatoxins, a property not previously studied. However, we have shown that desensitization of guinea pig ileum with rat anaphylatoxin fully cross-desensitizes to guinea pig anaphylatoxin, an observation fully supporting the original conclusions (22, 45).

It may now be stated with certainty, 56 yr after Friedberger's original discovery (23), that anaphylatoxin is indeed a product of the complement system. The biochemical characterization of its mechanism of generation and the chemical characterization of the product itself are directly approachable problems which have been under study in this laboratory. However, definition of the conditions under which anaphylatoxin may be formed in vivo and evaluation of its pathophysiological importance in the pathogenesis of inflammatory processes mediated by complement are perhaps the more formidable challenges for future investigations.

## SUMMARY

Interaction in free solution of highly purified preparations of human C'1 esterase, C'4, C'2, and C'3, in the presence of $Mg^{2+}$, resulted in rapid generation of an activity indistinguishable by biological criteria from anaphylatoxin. The formation of anaphylatoxin was associated with immunoelectrophoretic conversion of C'3 to anodically faster migrating proteins and was unaffected by the presence or absence of added C'5.

The biological properties of human anaphylatoxin prepared in this manner include: contraction and desensitization of isolated guinea pig ileum, failure to contract isolated rat uterus, enhancement of vascular permeability in guinea pig skin, degranulation of mast cells in guinea pig mesentery preparations, and liberation of histamine from suspensions of rat peritoneal mast cells. The smooth muscle–contracting and permeability enhancing properties were fully blocked by an antihistaminic drug, triprolidine. No cross-desensitizing activity on

guinea pig ileum was demonstrable between rat and human or guinea pig and human anaphylatoxins but a closer biological relationship between rat and guinea pig anaphylatoxins was observed.

It is concluded that anaphylatoxin is a product of the complement system. Its possible relationship to apparently similar activities currently being obtained in other laboratories has been discussed.

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