

Permeability-increasing activity in hereditary angioneurotic edema plasma: *II. Mechanism of formation and partial characterization*

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Permeability-Increasing Activity in Hereditary Angioneurotic Edema Plasma

II. MECHANISM OF FORMATION AND PARTIAL CHARACTERIZATION

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ABSTRACT Plasma from persons with hereditary angioneurotic edema readily developed the capacity to increase vascular permeability and to induce the isolated rat uterus to contract. Both activities resided in a small, heat-stable molecule that was apparently a polypeptide. Crude preparations of the polypeptide were inactivated during incubation with trypsin. They also failed to produce pain and erythema, but caused markedly increased vascular permeability in human skin. These characteristics differ from those of bradykinin, from which crude preparations of the polypeptide could also be distinguished by electrophoretic mobility and paper chromatographic behavior. Proof that the polypeptide is truly different from bradykinin must await its further purification. Histamine played no role in the activities observed.

Although the enzymes functioning to release the permeability factor and kinin activities in hereditary angioneurotic edema plasma were not clearly defined, one or more plasma enzymes other than C'1 esterase presumably participated either in conjunction with C'1 esterase or in *pari passu* events to release the polypeptide mediating these activities.

INTRODUCTION

Plasma and serum from persons with hereditary angioneurotic edema are deficient in the normal inhibition di-

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rected against C'1 esterase (1-5). This inhibition is a property of an alpha globulin of serum that also inhibits a globulin permeability factor (PF/Dil), plasma kallikrein (6, 7), a subcomponent of the first component of complement known as C'1r and plasmin (8). The deficiency in inhibition of C'1 esterase permits spontaneous formation of C'1 esterase in plasma from persons with hereditary angioneurotic edema, a tendency enhanced during episodes of swelling (4, 5).

The experiments to be described demonstrate that in addition to C'1 esterase, an agent that increases cutaneous vascular permeability evolves in plasma from persons with hereditary angioneurotic edema. Whereas this property was readily generated in plasma from patients in remission, its formation was enhanced in plasma during attacks of swelling. The active agent appeared to be a small polypeptide which also induced contraction of isolated smooth muscles, and thus behaved as a kinin. Its formation required the action of plasma enzymes other than C'1 esterase, for substances incapable of inhibiting C'1 esterase blocked its generation.

METHODS

Plasma from normal persons, affected members of several kindreds with hereditary angioneurotic edema, and others with angioneurotic edema which was not hereditary (i.e., those with normal serum inhibition of C'1 esterase) were separated from blood drawn into silicone-coated Lusteroid test tubes (Lusteroid Container Corp., Maplewood, N. J.) containing sodium citrate buffer (pH 5.2, 0.13 M with respect to citrate) alone, or mixed with Na₂H₂EDTA to provide a final EDTA concentration of 6×10^{-2} M in the blood. Plasma was recovered after centrifugation of blood at 2500 rpm in an International PR-2 refrigerated centrifuge, and rendered platelet-deficient by recentrifugation at 20,000 g

at 2°C for 15 minutes. All plasma samples used were carefully protected from contact with glass until such time as the enzymatic constituents of the plasma were inactivated by boiling. Hereditary angioneurotic edema plasma samples used in experiments to be described were from persons deficient in serum antigen that reacted with specific rabbit antiserum to normal inhibitor of C'1 esterase except where noted.

Soybean trypsin inhibitor (five times crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in 0.15 M sodium chloride at a concentration of 10 mg/ml, and diluted to 1.0 mg/ml in incubation mixtures and final assay mixtures measuring permeability factor activity. Heparin sodium (The Upjohn Co., Kalamazoo, Mich.) was used in incubation mixtures at final concentrations of 100 U/ml. In some experiments, heparin powder (sodium heparinate, The Upjohn Co., Kalamazoo, Mich., Lot No. 32-LLC-3) was used in final concentrations of 624 U/ml or 1000 U/ml in incubation mixtures. Hexadimethrine bromide (Polybrene, Abbott Laboratories, Chicago, Ill.) was dissolved in barbital-saline buffer at 1000 µg/ml and used in incubation mixtures at 100 µg/ml. Trasylol (compound A-128, proteinase inhibitor; FBA Medical Research Division of Metachem, Inc., New York), a kallikrein inhibitor, was diluted in buffer to concentrations of 200 U/ml of incubation mixtures. Epsilon aminocaproic acid (Mann Research Laboratories, New York) was dissolved in distilled water at 1.0 M concentrations and used during incubation at 0.1 M concentrations. Purified serum inhibitor of C'1 esterase was prepared by Dr. Jack Pensky of Western Reserve University, by modifications of a published method (9), and used in amounts to be noted after being dissolved in 0.15 M sodium chloride.

Bradykinin (Sandoz Pharmaceuticals, Hanover, N. J.), a synthetic nonapeptide, was dissolved in 0.15 M sodium chloride or in buffers noted in experiments. Kallidin (lysyl bradykinin, a decapeptide) was generously provided by Dr. E. Nicolaides (Research Laboratories, Parke Davis and Co., Ann Arbor, Mich.) and was diluted in buffers.

Vascular permeability was measured in the manner described by Miles and Wilhelm (10), using carefully depilated female albino guinea pigs as subjects. The experimental animals were given intravenous injections of mixtures of 5% Pontamine Sky blue 6X (E. I. DuPont de Nemours and Co., Wilmington, Del.) in amounts of 1.2 ml/kg body wt in 0.075 M sodium chloride mixed with an antihistaminic, triprolidine (Actidyl, Burroughs Wellcome, Co., London, 1.0 mg/ml of solution) to provide injection of 0.1 mg of triprolidine/kg body wt. The concentrations of triprolidine used abolished the increase in vascular permeability induced by injections of 2.0 µg of histamine (histamine diphosphate, Sigma Chemicals, St. Louis, Mo.), calculated as free base, which was used as a control in each guinea pig. Some animals were not given antihistamine to allow the detection of any histamine-like action of test samples. Samples of 0.1 ml of the mixtures to be tested for their effect upon vascular permeability were injected intracutaneously beginning a few minutes after the injection of Pontamine Sky blue. The ability of injected substances to increase vascular permeability was determined by measuring the size of blued skin lesions around injections after an interval of at least 15 min following the last test. The largest diameter of each spot and its perpendicular were measured and the mean value of the diameters calculated. In each experiment, four guinea pigs were given a single injection of each dilution of each sample. The diameters of lesions expressed represent the means of those in four guinea pigs for any one experiment. In 45 experiments the means diameter of lesions induced by

injections of buffer was 2.2 mm, and 2 sd = 1.4 mm. The standard error was 0.1 mm. Test samples were injected and lesions measured without knowledge of their contents.

The ability of test mixtures to induce contraction of isolated smooth muscles was usually measured on the horn of the uterus of a rat that had been given an intraperitoneal injection of 10 µg/100 gm of body wt of diethylstilbestrol (Eli Lilly & Co., Indianapolis, Ind., 5 mg/ml in oil), which was diluted in olive oil (McKesson & Robbins, Bridgeport, Conn.) to induce estrus. The uteri of Sprague-Dawley rats that weighed 200–250 g were suspended in an 8.0 ml organ bath in de Jalon's mixture (11). Muscle contractions were recorded on a kymograph with electrosensitive paper, using an electrically stimulated lever arm that was attached to the muscle with nonconducting thread, or with a lever attached to a differential transformer (12) (Phipps & Bird, Richmond, Va.) and a Texas Instrument recorder. When guinea pig ileum or rat duodenum was used, the temperature of the organ bath was raised to 37°C; assays with the rat uterus were done from 29° to 31°C. The effectiveness of a test mixture was a function of the height of the concentration, recorded in mm in the experiments. The rat uterus preparations were sensitive to 2 ng of bradykinin in the organ bath (2×10^{-7} M in assay).

Trypsin (crystalline, salt-free, Worthington Biochemical Corp., Freehold, N. J., Lot No. TL-44-14) was dissolved in 0.1 N hydrochloric acid at 500 mg/ml, stored at -65°C, and for use in experiments was further diluted in the buffer to be used immediately before use to give a final concentration of 50 µg/ml of incubation mixture. Carboxypeptidase B (Worthington Biochemical Corp., batch No. 6089) was diluted in 0.15 M sodium chloride in an initial concentration of 12 mg/ml and samples of the enzyme were then mixed with their volumes of 10^{-2} M cobalt chloride. Further dilutions were then carried out in the buffers to be used in experiments. Final concentrations in incubation mixtures testing the effect of carboxypeptidase B on crude permeability factor preparations were 10.8 µg/ml. This amount did not increase vascular permeability in the assay used. Alpha chymotrypsin (Worthington Biochemical Corp., three times crystallized, batch No. CD1 6142-3) was also dissolved in solutions of 0.15 M sodium chloride in high concentrations for storage at -65°C, and was diluted further for use in the buffer used in a particular experiment, so that it was in a concentration of 50 µg/ml of incubation mixtures. This enzyme was inactivated by boiling just before assay of these mixtures, for it had a pronounced effect on vascular permeability. Each enzyme was incubated at 37°C for 30 min with 10 volumes of the agents to be tested: crude preparations of permeability factor from hereditary angioneurotic edema plasma, solutions of synthetic kinins or plasma. Mixtures containing plasma were incubated in silicone-coated Lusteroid tubes; those containing crude permeability factor were incubated in polystyrene tubes (Falcon Plastics, Los Angeles, Calif.). After incubation, the mixtures were tested for permeability-increasing and kinin-like properties. Trypsin digestion mixtures were incubated at pH 7.6–7.8 in phosphate or Tris buffer; alpha chymotrypsin and carboxypeptidase B digestion mixtures were incubated at pH 7.4.

In experiments testing permeability properties of solutions, dilutions were carried out in barbital-saline buffer, at pH 7.4 and ionic strength 0.15, prepared by dissolving 2.06 g of sodium barbital, 2.76 g of barbital, and 7.8 g of sodium chloride in 1 liter of distilled water. De Jalon's solution (11) contained 9.0 g of sodium chloride, 0.4 g of potassium chloride, 0.03 g of calcium chloride, 0.15 g of sodium bicarbonate, and 1 g of glucose in 1 liter of distilled water.

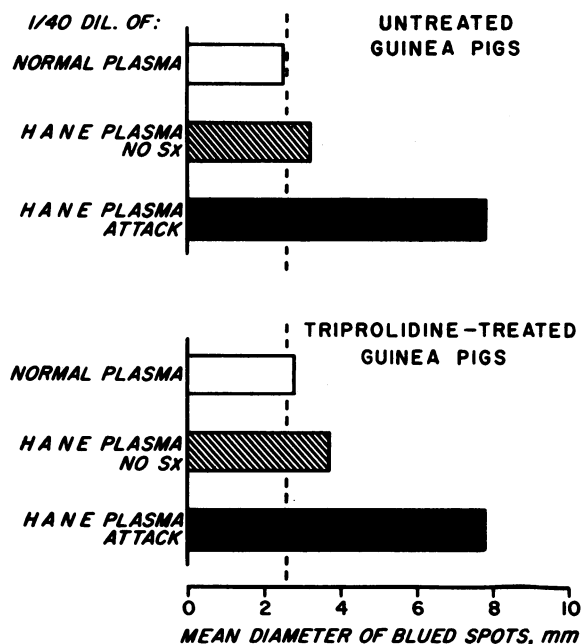


FIGURE 1 The bars illustrate the effects of plasma samples upon vascular permeability in a typical experiment. Shown are the diameters of the lesions surrounding injections of 0.1 ml of dilutions of each sample into each of three guinea pigs which had not received antihistaminic medication (top) and three which had received an antihistaminic, triprolidine (bottom). Plasma samples from a normal person, from a patient with hereditary angioneurotic edema in remission (HANE, no Sx), and from the same patient having an attack of edema were diluted 1/10, 1/20, 1/40, and 1/80 in barbital-saline buffer in silicone-coated tubes immediately before injection. The effects of the 1/40 dilutions are shown as mean values, determined as described. The broken line indicates the effect of injections of 0.1 ml of buffer used as diluent.

When materials were diluted for assay for their activity on smooth muscle, 0.067 M sodium phosphate buffer, pH 7.4, was usually used; in some instances barbital-saline buffer at the same pH was used. In preparing dilutions of substances for permeability assay, any inhibitor used in an incubation mixture was added to the buffer used as diluent for assay of samples tested at 1/10 through 1/80 to give concentrations equal to those in incubation mixtures. Silicone-coated Lusteroid tubes were used to prepare dilutions of plasma. In paper electrophoresis experiments, a pH 8.6, 0.05 M veronal buffer as described by Laurell, Laurell, and Skoog (13) was used. Electrophoresis was carried out for 8 hr in a Durrum cell at 150 v. During solvent chromatography, No. 3 Whatman filter paper was used.

RESULTS

A. Generation of permeability factor activity in plasma.

Plasma samples, harvested in silicone-coated vessels and carefully protected from contact with glass, were thawed for the first time after storage at -65°C , doubly diluted from 1/10, through 1/80, in silicone-

coated Lusteroid tubes, and injected intradermally into guinea pigs without delay to avoid generation of PF/Dil. Plasma from persons having bouts of hereditary angioneurotic edema (*attack plasma*) markedly increased vascular permeability as shown by the size of lesions surrounding injections, and the intensity of bluing. A typical experiment is illustrated in Fig. 1. This effect was not blocked by prior treatment of experimental animals with an antihistamine, triprolidine (Fig. 1). Plasma samples from persons known to have hereditary angioneurotic edema, but in remission at the time of phlebotomy (*remission plasma*), had a minimal effect upon vascular permeability, as demonstrated in Fig. 1. Injections of normal plasma similarly diluted, had no more effect upon permeability than buffer (Fig. 1). When 1/10 dilutions of normal plasma were tested, vascular permeability was usually significantly increased, but this effect became minimal with dilution to 1/20 or more, in contrast to the persistent and pronounced effect of attack plasma from persons with hereditary angioneurotic edema at dilutions of 1/80 or sometimes even more. The mean diameter of blued lesions induced by plasma samples from 16 normal persons, diluted 1/40, was only 3.2 mm, even after incubation of the plasma for 3 hr before testing. In contrast, 15 samples of attack plasma from persons with hereditary angioneurotic edema, diluted 1/40, induced lesions with a mean diameter of 6.6 mm (Table I). Samples of remission plasma were more variable, perhaps reflecting the difficulty in being certain that the subject was truly free of edema at the time. When seven different remission plasma samples, freshly thawed, were diluted 1/40 and injected, the mean diameter of the resulting sets of blued lesions was 4.1 mm, but a large group of citrated remission plasma samples that had been incubated with EDTA at 0° or 37°C contained greater permeability activity (Table I). Plasma from 10 persons having bouts of angioneurotic edema that was not hereditary, incubated with EDTA and similarly diluted for assay, induced blued lesions having a mean diameter of only 2.6 mm (Table I).

The effect of incubation on the formation of permeability factor activity in vitro in remission plasma was further illustrated in individual samples tested before and after incubation at 37°C (Table II). When blood from a patient in remission was drawn into purified human serum inhibitor of C'1 esterase, the plasma recovered from this sample failed to form increased permeability activity during incubation, whereas that derived from blood drawn simultaneously from the same person into sodium chloride developed this property (Table II, bottom).

When citrated attack plasma was incubated at 37°C , its permeability-increasing property deteriorated. How-

TABLE I
Permeability Factor Activity of Plasma Samples Diluted 1/40

Sample	No. tested	Diameters of lesions			
		0 min		180 min at 37°C	
		Mean	Range	Mean	Range
				<i>mm</i>	
Buffer	45	2.2	1.0-3.0	—	—
Bradykinin, 8.0 ng injected	12	7.8	6.0-9.0	—	—
Bradykinin, 1.0 ng injected	3	5.2	4.9-5.9	—	—
Bradykinin, 0.5 ng injected	3	2.8	2.6-3.0	—	—
HANE attack plasma	15,14	6.6	4.3-8.1	6.0	4.0-8.3
HANE remission plasma	7,27	4.1	2.8-7.8	6.1	4.0-8.6
Normal plasma	3,16	3.2	2.8-3.8	3.2	1.9-4.4
Angioneurotic edema plasma (not hereditary)	10	—	—	2.6	1.0-3.8

In these experiments, EDTA plasma samples, diluted in silicone-coated vessels to 1/10, 1/20, 1/40, and 1/80 just before assay were injected intradermally into each of four guinea pigs previously given Pontamine Sky blue and triprolidine intravenously. The mean diameters of the lesions induced by the 1/40 dilution of each sample in each guinea pig were averaged with mean diameters obtained with the same dilution in other experiments with different plasma samples. Where two sets of numbers are given to show the number of samples tested, the first indicates the number tested without incubation, and the second the number tested after incubation. Usually, samples tested before and after incubation at 37°C × 3 hr in siliconized test tubes were not identical.

ever, if it was mixed with EDTA to give concentrations of 6×10^{-8} M, either upon phlebotomy or immediately following initial thawing of a plasma sample, the permeability property was preserved during incubation, and sometimes increased. Occasionally, attack plasma failed to induce increased vascular permeability and it failed to develop any activity during incubation in a silicone-coated test tube *in vitro*, as if the generating mechanism had been exhausted.

Since permeability factor activity could be generated *in vitro* in remission plasma, the ability of some inhibitors of plasma enzymes to block its formation was tested. When soybean trypsin inhibitor was mixed with a sample of remission plasma before incubation, the formation of permeability factor activity during incubation was blocked (Fig. 2). This inhibitor blocked the generation of permeability factor activity in three other remission plasma samples which could be tested. Once generated, the permeability activity could not be blocked by the soybean trypsin inhibitor, as illustrated in Fig. 2. The addition of inhibitor of C1 esterase to another remission plasma at a concentration of only 1 U/ml (i.e., less than 20% of normal plasma concentration) before incubation blocked the formation of permeability factor activity, while it, too, was ineffective if added after incubation, when activity had already been generated (Fig. 2). Generation of activity was blocked when three other plasma samples from patients in remission were so tested. When a high concentration of heparin (final con-

centrations of 624 U/ml) was mixed with remission plasma that had been stored at -65°C, the generation of permeability factor activity during incubation of the mixture was not impaired (Table III). However, if blood was drawn from a patient in remission directly into EDTA and heparin, to give a final concentration of only 100 U/ml, and a matched sample drawn into sodium citrate and EDTA, during subsequent incubation of plasma from these mixtures even after freezing, the development of permeability factor activity in the heparinized plasma was blocked (Table III). Therefore, in the absence of heparin some change had apparently occurred during freezing and thawing of the plasma which favored the generation of the permeability property. Hexadimethrine bromide did not inhibit the generation of permeability factor properties *in vitro* in remission plasma. Its effect on plasma not previously frozen was not tested.

Mixtures of trasylol (200 U/ml of mixture) and remission plasma developed nearly as much permeability factor activity during incubation as did mixtures of buffer and remission plasma. When epsilon aminocaproic acid and EDTA plasma of a patient with hereditary angioneurotic edema in remission were incubated together, there was no apparent impairment of the generation of permeability factor activity. Partial inhibition could not be excluded because epsilon aminocaproic acid itself, in concentrations of 0.1 M in buffer, increased vascular permeability (Table III).

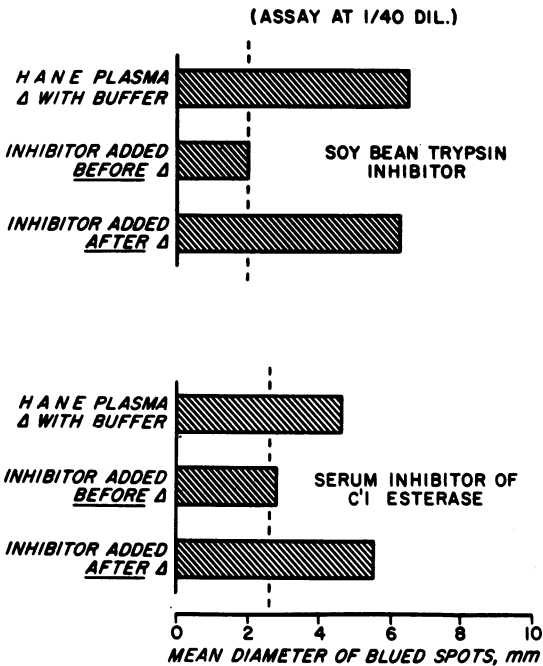


FIGURE 2 The effect of the addition of inhibitors to two hereditary angioneurotic edema plasma samples before and after incubation (Δ) in vitro at 37°C is compared with the effect of incubation of each plasma with buffer in place of inhibitor. The soybean trypsin was added to plasma from a different patient than the inhibitor of C'1 esterase in these experiments. Each incubation mixture was diluted 1/10, 1/20, 1/40, 1/80 just before assay in which 0.1 ml of each dilution of each mixture was injected into each of four guinea pigs which had received triprolidine. The mean values of the diameters of these lesions were determined as described; only the effects of the 1/40 dilution are shown. In each experiment, the lower bar shows the effect of a mixture to which the inhibitor was at the end of the incubation period but just before dilution for assays. The vertical broken lines indicate the effects of barbitol buffer used as diluent in each experiment.

B. Partial characterization of permeability factor activity. When hereditary angioneurotic edema plasma containing permeability factor activity was extracted with ether, and the ether layer removed, there was no loss of permeability factor activity from the aqueous phase of the plasma. When the ether layer was dried, and the residue redissolved in barbital-saline buffer in a volume equal to that of the original plasma, the ether fraction had no permeability factor activity. Hereditary angioneurotic edema plasma with permeability-increasing properties was extracted with 0.3 N trichloroacetic acid, the acid layer removed and extracted with ether to free it of trichloroacetic acid. When this acid extract was concentrated, and redissolved in barbital-saline buffer, it failed to increase vascular permeability, as if the mediating substance were not acid-soluble. As the ma-

terial precipitated with the acid was not tested, it is not clear if the activity had been precipitated or destroyed. The mediating substance seemed to be a relatively small molecule, for when hereditary angioneurotic edema plasma was dialyzed overnight against an equal volume of buffer, the dialyzed plasma and perfusate then contained approximately the same concentrations of permeability factor activity.

In spite of the absence of the permeability-increasing property from acid extracts of plasma, it was exceedingly heat stable. When a sample of hereditary angioneurotic edema plasma was placed in boiling water for 30 min, and the plasma coagulum then minced and extracted twice with $\frac{1}{2}$ the original plasma volume of aqueous buffer, from 50 to 90% of the permeability factor activity could be recovered in the buffer. The crude preparations of permeability factor obtained by this means, filtered once through 0.45 μ Millipore filters, provided excellent preparations for partial characterization of the mediating substance. When normal plasma or samples from persons with angioneurotic edema

TABLE II
In Vitro Generation of Permeability Factor Activity in Individual Samples of Remission Plasma: Effect of Purified Serum Inhibitor of C'1 Esterase (EI)

Sample	Dilution	Mean diameter of lesions	
		0 minutes	180 min at 37°C
		<i>mm</i>	
Remission plasma No. 1	1/80	3.6	7.3
Remission plasma No. 2	1/80	2.8	4.8
Remission plasma No. 3	1/80	2.0	5.8
Remission plasma No. 4	1/80	2.3	5.8
Remission plasma from blood drawn into EI to give 3.0 U/ml	1/80	3.5	4.0
Remission plasma from blood drawn into sodium chloride	1/80	3.4	5.8

Samples of plasma from four different persons with HANE in remission were tested when freshly thawed by diluting each sample in barbital saline buffer to 1/10, 1/20, 1/40, and 1/80 and injecting 0.1 ml of each dilution from each patient intradermally into each of four guinea pigs. The mean diameters of the lesions induced by the same plasma samples similarly diluted and tested after incubation at 37°C for 3 hr are shown. The last two samples (not numbered) were from another patient in remission. At the same phlebotomy of this patient, some of the blood was drawn into purified human serum inhibitor of C'1 esterase in sufficient quantity to give 3.0 U/ml, and some of it into an equal volume of 0.15 M sodium chloride. These two samples were incubated and tested as described above.

TABLE III
Effect of Some Inhibitors upon the Generation of Permeability Factor Activity in Hereditary Angioneurotic Edema Plasma (HANE Plasma)

HANE plasma, dilution 1/40	Inhibitor in incubation mixture	Diameter of lesion
	<i>37° × 180 min</i>	<i>mm</i>
Remission plus buffer	0	5.8
Remission plus buffer	Trasylol, 100 U/ml mixture	5.3
Remission plus buffer	0	7.1
Remission plus buffer	Hexadimethrine bromide, 100 µg/ml mixture	7.6
Remission plus buffer	Hexadimethrine bromide added after incubation, before assay	7.5
Remission plus buffer	0	7.4
Remission plus buffer	Heparin, 624 U/ml mixture	6.0
Remission plus buffer	Heparin, 624 U/ml mixture added after incubation, before assay	6.5
Remission, blood drawn into heparin initially	Heparin, 100 U/ml mixture	3.5
Remission, no heparin at phlebotomy	0	7.9
Remission plasma plus buffer	0	7.6
Remission plasma plus buffer	Epsilon aminocaproic acid 0.1 M in mixture	7.0
Buffer	Epsilon aminocaproic acid 0.1 M in mixture	4.4
Buffer controls	—	1.8–2.5

which was not inherited were heated and extracted in this manner, usually no measurable permeability factor activity was recovered.

When crude preparations of permeability factor in extracts of boiled hereditary angioneurotic edema plasma were applied to a rat uterus, as little as 0.01 ml of a $\frac{1}{10}$ dilution could induce the muscle to contract. Similar preparations from normal plasma induced contractions only if 0.1 or 0.2 ml of undiluted material was used. Samples of crude permeability factor from hereditary angioneurotic edema plasma also caused the rat duodenum to lengthen, and in high concentrations, induced the guinea pig ileum to contract. Tachyphylaxis was not observed. The effects of the crude permeability factor upon these muscle preparations resembled that of bradykinin (14).

If the substance mediating permeability change was bradykinin, it should have hypotensive effects in an intact animal (15). When 0.1 ml samples of the dialyzable components of the crude permeability factor were injected intravenously into anaesthetized rats, instead of lowering blood pressure, a mild pressor effect was observed unassociated with tachyphylaxis. Whereas the pressor action may have been due to a substance different from the permeability factor, the lack of any depressor effect of the dialyzable crude permeability factor contrasted with the marked depressor action of 0.3 µg

of synthetic bradykinin. However, when bradykinin was added to normal siliconed plasma and EDTA, the mixture boiled and extracted as if in preparation of crude permeability factor, the dialyzable fractions had only slight or no depressor effect on blood pressure, even though they were rich in kinin activity.

After electrophoresis of bradykinin and of concentrated crude permeability factor from hereditary angioneurotic edema plasma at pH 8.6 on paper strips, the strips were dried and divided transversely into 1 cm segments. Each segment was eluted in 1.0 ml of barbital-saline buffer, pH 7.4, and these eluates were tested for their kinin activity on the isolated rat uterus. The electrophoretically separated fractions of bradykinin were more electropositive than those of the crude permeability factor. The peak of the bradykinin activity was from 7 to 8 cm to the cathodal side of the origin, and that of the permeability factor preparation was 5 cm from the origin in the same direction.

The mobility of crude permeability factor preparations and of bradykinin were compared in solvent chromatography on filter paper. Crude permeability factor that had been extracted from boiled hereditary angioneurotic edema plasma with distilled water was concentrated by lyophilization, redissolved in distilled water, and subjected to ascending paper chromatography for 16 hr in mixtures of four parts redistilled butanol, one part

TABLE IV
Effect of Proteolytic Enzymes on Permeability Factor Activity in Hereditary Angioneurotic Edema (HANE) Plasma

Test sample	Amount injected	Permeability activity Diameter of blue lesions
<i>Incubated at 37°C × 30 min</i>		
HANE plasma plus buffer	0.1 ml, 1/20 dilution	9.0
HANE plasma plus trypsin (50 µg/ml); SBTI added after incubation	0.1 ml, 1/20 dilution	4.4
HANE plasma plus SBTI and trypsin; SBTI added before trypsin	0.1 ml, 1/20 dilution	7.2
HANE plasma plus carboxypeptidase B, (10.8 µg/ml)	0.1 ml, 1/20 dilution	3.0
Buffer	0.1 ml	2.2

HANE plasma samples were obtained at the same phlebotomy from a person with arm and hand swelling. The plasma samples were incubated at 37°C for 30 min with the enzymes, in the final concentrations noted, in silicone-coated Lusteroid test tubes with soybean trypsin inhibitor (SBTI), 1 mg/ml, or buffer. Samples were diluted in cold buffer in silicone-coated test tubes from 1/10 through 1/80 immediately before injection of 0.1 ml samples into guinea pigs which had received triprolidine intravenously. Dilutions of 1/20 demonstrated differences more clearly than those of 1/10, and are shown.

glacial acetic acid, and five parts distilled water. Synthetic bradykinin was similarly treated. Both sets of strips were then dried, divided transversely into 1 cm segments, and each segment eluted into 1.0 ml of barbital-saline buffer, or 0.15 M sodium chloride. When these eluates were tested for their kinin content, the rate of flow of bradykinin activity with respect to the solvent front was 0.425, whereas that of the kinin from hereditary angioneurotic edema plasma was 0.290 or less. No activity was found in any of the fractions derived from normal plasma. When bradykinin was added to *normal* plasma before boiling, the relative rate of flow of the kinin activity then recovered from chromatographic strips varied from 0.310 to 0.280, and thus closely resembled that of the kinin from hereditary angioneurotic edema plasma ($R_f = 0.280$). The kinin activity from these chromatograms of extracts of hereditary angioneurotic edema plasma was not inhibited by soybean trypsin inhibitor or by highly purified serum inhibitor of C'1 esterase. It was largely inactivated by trypsin.

The permeability factor and kinin properties in hereditary angioneurotic edema plasma and in the crude preparations of permeability factor derived therefrom could be destroyed by several proteolytic enzymes, as if the mediating substance were a polypeptide. When plasma from a patient was incubated with carboxypeptidase B or trypsin at 37°C for 30 min, its permeability factor function was lost (Table IV). When the *crude preparations of permeability factor* derived from hereditary angioneurotic edema plasma were so treated, both kinin and permeability factor activities were lost. In the experiments shown in Table V, trypsin, alpha chymotryp-

sin, and carboxypeptidase B all destroyed the kinin activity of the preparation. When five times larger amounts of the preparation that had been treated with these enzymes were applied to the muscles, an effect could be noted in the trypsin-treated preparation but not in the other two. In six other experiments, 80% or more of kinin activity in different crude preparations of permeability factor was inactivated during incubation with trypsin. Neither bradykinin or kallidin lost either property during incubation with the same concentrations of this trypsin preparation (Table V). Both of these polypeptide kinins are known to be susceptible to inactivation by carboxypeptidase B (16) and alpha chymotrypsin (17) and these enzymes were not specifically tested in the experiments shown in Table V. Even when bradykinin was mixed with normal plasma, the mixture boiled and extracted, as in preparation of crude permeability factor, the kinin activity recovered was totally resistant to the action of trypsin.

When two normal humans, who had received 5 ml of Evans blue intravenously, were given intradermal injections of crude kinin-permeability factor from hereditary angioneurotic edema plasma, vascular permeability was increased, despite the fact that they had received 125 mg of pyribenzamine 90 min earlier. A 200 ng sample of synthetic bradykinin injected into the same subjects induced areas of slight swelling, which were not, however, notably blued. The prominent feature of this reaction was the delayed onset of transient but searing pain in the area; there was also surrounding erythema, but no itching or pseudopod formation. An extract was prepared from siliconized EDTA plasma from normal

TABLE V

Effect of Proteolytic Enzymes on Kinin and Permeability Factor ("PF") from Hereditary Angioneurotic Edema (HANE) Plasma and Synthetic Bradykinin and Kallidin

Test sample	Dose	Kinin activity		Dose	Permeability activity
		Rat uterus contraction	Rat duodenum lengthened		Mean diameter of blue lesions
<i>Incubated at 37°C × 30 min</i>		<i>millimeters</i>			<i>millimeters</i>
10 ⁻⁶ M bradykinin plus buffer	0.05 ml	34	—	0.1 ml	8.5
10 ⁻⁶ M bradykinin plus trypsin, 50 µg/ml; SBTI added after incubation	0.05 ml	38	—	0.1 ml	7.8
10 ⁻⁶ M bradykinin plus SBTI and trypsin; SBTI added before trypsin	0.05 ml	40	—	0.1 ml	8.5
10 ⁻⁶ M kallidin plus buffer	0.05 ml	37	—	10 ⁻⁸ M, 0.1 ml	8.5
10 ⁻⁶ M kallidin plus trypsin, 50 µg/ml; SBTI added after incubation	0.05 ml	41	—	10 ⁻⁸ M, 0.1 ml	8.5
10 ⁻⁶ M kallidin plus SBTI and trypsin SBTI added before trypsin	0.05 ml	42	—	10 ⁻⁸ M, 0.1 ml	—
Crude "PF" plus buffer	0.02 ml	72	20	1/10 Dilution, 0.1 ml	5.6
Crude "PF" plus trypsin, 50 µg/ml; SBTI added after incubation	0.02 ml	0	0	1/10 Dilution, 0.1 ml	3.9
Crude "PF" plus SBTI and trypsin; SBTI added before trypsin	0.02 ml	53	—	1/10 Dilution, 0.1 ml	6.6
Crude "PF" plus carboxypeptidase B, 10.8 µg/ml	0.02 ml	0	0	1/10 Dilution, 0.1 ml	2.8
Crude "PF" plus alpha chymotrypsin, 50 µg/ml; mixture boiled 4 min after incubation	0.02 ml	0	0	1/10 Dilution, 0.1 ml	3.1
Bradykinin, 10 ng/assay	—	73	20	0.1 ml	5.1
Buffer	0.02 ml	0	0	0.1 ml	2.8

Two sets of experiments showing the effect of some enzymes on kinin and permeability factor activities are shown. In the experiment with synthetic bradykinin and kallidin, solutions of each were incubated at 37°C for 30 min in plastic test tubes with trypsin in the final concentration noted and soybean trypsin inhibitor (SBTI), 1 mg/ml of incubation mixture, or buffer. To measure kinin activity, variable volumes of these incubation mixtures were applied to the rat uterus to determine the smallest volume of untreated polypeptide that gave a maximal contraction of this muscle preparation. The kallidin was more effective than bradykinin in increasing vascular permeability. The guinea pigs had received triprolidine intravenously before the assays for permeability.

The lower part of the table shows the effect of crude "PF" prepared from plasma rich in this activity from a patient with HANE from which any sediment was removed by filtration. The enzymes tested were mixed with samples of "PF" to give final concentration noted. Both trypsin and alpha chymotrypsin markedly increased vascular permeability if injected before inactivation; SBTI (1 mg/ml) inactivated trypsin, and boiling 4 min inactivated alpha chymotrypsin in incubation mixtures. Carboxypeptidase B had no activity in either assay in concentrations used. A different rat uterus preparation was used than in experiments above testing synthetic kinins, which accounts for the differences in heights of maximal contractions with respect to permeability activity. The rat duodenum preparation used responded to acetyl choline with an immediate contraction, and to synthetic bradykinin with gradual lengthening.

persons to which bradykinin was added before the mixture was boiled and extracted, just as if to prepare crude kinin from hereditary angioneurotic edema plasma. When 0.1 ml of a 1/50 dilution of this material was injected intradermally into the normal subjects, a swelling of 6 mm was induced without blueing or erythema. There was also a delayed pain reaction. When the crude extract from hereditary angioneurotic edema plasma, with similar kinin concentration, was diluted 1/50 and injected in the same volume, a 9 mm swelling resulted with moderate (2+) blueing. There was no pain, erythema, itching, or pseudopod formation after this injection into the normal subjects even when they had not previously received an antihistamine. All samples injected were diluted in Veronal-sodium chloride buffer, pH 7.5, containing 1.5×10^{-4} M calcium and 5×10^{-4} M magnesium.

In the experiments illustrated in the tables and figures, plasma from persons with hereditary angioneurotic edema who had an inherited deficiency of functional serum inhibitor of C'1 esterase, as well as of serum antigen which reacted with a specific rabbit antiserum to normal human inhibitor, were used. In other experiments not shown, permeability factor activity was generated in plasma from affected members of two kindreds who had no measurable functional serum inhibitor of C'1 esterase, but who had a normal concentration of serum antigen resembling that of the normal serum inhibitor (cross-reactive material). The crude permeability factor prepared from their plasma was also heat stable, dialyzable, and had properties of a kinin.

DISCUSSION

The permeability-increasing property found in high concentration in plasma obtained from persons having bouts of hereditary angioneurotic edema (attack plasma) had activities resembling those of kinins rather than of histamine or histamine-releasing agents. The effect of the permeability-enhancing agent or agents was unimpaired in animals given doses of antihistamine sufficient to block the effects of 5.5 μ gm. of histamine diphosphate. Moreover, the activity of crude preparations of the permeability factor was readily demonstrable by its effect upon the isolated rat uterus, which is sensitive to kinins but insensitive to histamine or to an anaphylatoxin that releases histamine (18). Little effect was observed upon the isolated guinea pig ileum, known to be very sensitive to histamine (19). Its action in human skin differed from a response mediated by histamine, and was unimpaired in subjects given an antihistamine. These observations distinguish the permeability factor and kinin-like activity derived from hereditary angioneurotic edema plasma from the anaphylatoxins formed

by the interaction of purified C'1 esterase with purified preparations of C'4, C'2, and C'3 (20), and C'5 (21-23).

The kinin and permeability-increasing activities which formed in hereditary angioneurotic edema plasma apparently resided in the same small polypeptide molecule. Both properties were dialyzable and heat stable, and were inactivated by carboxypeptidase B, alpha chymotrypsin, and trypsin. In the absence of EDTA, the permeability-enhancing activity was readily lost from hereditary angioneurotic plasma, suggesting that it may have been destroyed by a plasma carboxypeptidase which can inactivate bradykinin and is known to require certain divalent cations for its action (24, 25). Direct proof that this plasma carboxypeptidase inactivates the agent derived from hereditary angioneurotic edema plasma is not yet available.

Whereas the kinin-permeability factor described had properties resembling those of kallidin and bradykinin, it differed from either of these in being subject to the proteolytic activity of a preparation of trypsin, which had no effect upon the two synthetic polypeptide kinins. Unlike these synthetic kinins, crude preparations of kinin-permeability factor from hereditary angioneurotic edema plasma failed to induce hypotension in the intact rat. However, the hypotensive effects of comparable fractions of mixtures of bradykinin and normal plasma were less clear than those of the pure polypeptide preparations. The kinin activity in the crude fractions of hereditary angioneurotic edema plasma had a different electrophoretic mobility than bradykinin, and there were suggestive differences in the behavior of these preparations during solvent chromatography on paper. Furthermore, when comparable preparations of the kinin-permeability factor from hereditary angioneurotic edema plasma and of mixtures of bradykinin and normal plasma were given intradermally to normal humans, their effects differed; the material from hereditary angioneurotic edema plasma induced a large, but painless area of swelling and blueing without erythema, and that containing bradykinin produced less swelling, no notable blueing, delayed pain, and surrounding erythema. Thus, our experiments suggest that a final mediator of kinin and permeability factor activity in hereditary angioneurotic edema plasma might be distinct from bradykinin or kallidin. The techniques used to differentiate among the various kinins were not, however, chemically definitive and the significance of these apparent differences must await further purification of the kinin-permeability factor.

C'1 esterase as well as kinin and permeability factor can be generated in remission plasma obtained from patients with hereditary angioneurotic edema (4). The generation of both activities can be inhibited by soybean trypsin inhibitor, which does not block the action of C'1 esterase, but can inhibit other plasma enzymes (26).

Heparin, known to impair generation of PF/Dil (27), could inhibit the formation of permeability factor activity. Since heparin had no effect if added to hereditary angioneurotic edema plasma that had been frozen and thawed, but that was inhibitory if added to the blood at phlebotomy, there must be an effect of freezing and thawing upon the plasma mechanisms leading to formation of the permeability factor. C'1 esterase was also more readily generated in hereditary angioneurotic edema plasma obtained during remission after repeated freezing and thawing (26), but the reasons for these effects are unclear. Even so, heparin impaired generation of C'1 esterase in remission plasma (28).

Epsilon aminocaproic acid does not block the action of C'1 esterase¹ but may impair its activation in hereditary angioneurotic edema plasma (26) and upon a sensitized cell (29). It can inhibit the activation of plasminogen (30, 31). Because of its own ability to increase vascular permeability in the present experiments, its effect upon the generation of permeability-increasing activity in hereditary angioneurotic edema plasma was unclear. Epsilon aminocaproic acid was reported to induce kinin release in preactive plasma (32), and to cause arteriolar dilatation followed by venous constriction in the dog (33). Lundh et al. (34) found that a patient with severe hereditary angioneurotic edema was relieved of attacks of swelling while taking epsilon aminocaproic acid. Possibly, this compound had a prophylactic effect by inhibiting the formation of one or more mediators of these symptoms in vivo. It is possible that the plasminogen system might act in some processes related to formation of swellings in hereditary angioneurotic edema (1, 4).

Neither soybean trypsin inhibitor nor purified human serum inhibitor of C'1 esterase inhibited permeability factor activity already formed in hereditary angioneurotic edema plasma, but either one could block its formation in remission plasma. Soybean trypsin inhibitor blocks the action of plasma kallikrein (35), plasmin (36), some stages of clotting (37), and the function of a globulin permeability factor sometimes called "PF/Dil" (38), but not C'1 esterase (39). Kagan and Becker (6, 7) found that preparations of purified human serum inhibitor of C'1 esterase can inhibit the functions of preparations of plasma kallikrein and of globulin permeability factor. Since the active permeability factor in hereditary angioneurotic edema plasma was not itself inhibited by either soybean trypsin inhibitor or inhibitor of C'1 esterase, the final mediator in the plasma differs from plasma kallikrein, globulin permeability factor, kallikrein, and C'1 esterase. Any or all of these substances, however, might participate in intermediate steps in its formation. The possibility that kallikrein and

¹ Donaldson, V. H. 1964. Unpublished observations.

PF/Dil may participate in events leading to enhanced generation of C'1 esterase in hereditary angioneurotic edema plasma was suggested recently (26, 28). In other experiments, the permeability properties of normal plasma and hereditary angioneurotic edema plasma, diluted 1/250 and 1/500 and mixed with glass, were quantitatively identical.² We were thus unable to show differences in PF/Dil formation in these plasma samples by this method. If PF/Dil were already active in undiluted hereditary angioneurotic edema plasma, its activity would probably persist during dilution so that its effect would equal that of diluted normal plasma. Thus, its participation has not been ruled out.

The final mediator of kinin and permeability factor activities in hereditary angioneurotic edema plasma behaved as if it were a polypeptide that is perhaps distinct from bradykinin or kallidin. Since these two kinins are generated by kallikreins, it is possible that some other plasma mechanism acts to form one or more kinin-permeability factors in this plasma. Trasylol, a kallikrein inhibitor (40, 41), was ineffective in preventing formation of permeability activity in this plasma, also suggesting that other enzymes may function. One alternative possibility has been suggested by the studies of Klemperer, Donaldson, and Rosen (42). Their experiments indicated that purified human C'1 esterase increased vascular permeability in human skin in a manner independent of histamine release, as reported presently, but apparently dependent upon C'2. Persons with a severe inherited deficiency of C'2 failed to demonstrate significantly increased vascular permeability following intradermal injections of purified C'1 esterase (42). Possibly, then, a polypeptide kinin with permeability factor activity is released from C'2 by C'1 esterase. Mayer and his associates (43, 44) have described the fragmentation of C'2 during complement action. Studies of Eisen (45) and Armstrong and Mills (46) have also indicated that there may be kinin-forming systems in normal plasma that differ from kallikrein and which may release polypeptides different from bradykinin or kallidin. Evaluation of more highly purified preparations of kinin-permeability factor from hereditary angioneurotic edema plasma will clarify the relationship between this material and the polypeptide kinins currently recognized.

A role for plasma kallikrein in hereditary angioneurotic edema was originally suggested by studies of Landerman, Webster, Becker and Ratcliffe (47) and also by Burdon, Queng, Thomas and McGovern (48). The latter group described decreased plasma kininogen (kinin precursor, or kallikrein substrate) in plasma obtained from patients who had just had attacks of hereditary angioneurotic edema. Perhaps molecules other than

² Ratnoff, O. D., and V. H. Donaldson. 1966. Unpublished observations.

kallidin and bradykinin can be released from this kinogen which have kinin and permeability factor activity. It is also possible that more than one factor may mediate the activity in unfractionated hereditary angioneurotic edema plasma. Possibly a heat labile mediator also exists. If so, it was rendered nonfunctional with respect to vascular permeability by treatment of plasma with proteolytic enzymes.

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REFERENCES

- Donaldson, V. H., and R. R. Evans. 1963. A biochemical abnormality in hereditary angioneurotic edema. Absence of serum inhibitor of C'1 esterase. *Amer. J. Med.* **35**: 37.
- Levy, L. R., and I. H. Lepow. 1959. Assay and properties of serum inhibitor of C'1 esterase. *Proc. Soc. Exp. Biol. Med.* **101**: 608.
- Donaldson, V. H. 1966. Serum inhibitor of C'1 esterase in health and disease. *J. Lab. Clin. Med.* **68**: 369.
- Donaldson, V. H., and F. S. Rosen. 1964. Action of complement in hereditary angioneurotic edema. The role of C'1 esterase. *J. Clin. Invest.* **43**: 2204.
- Austen, K. F., and A. L. Sheffer. 1965. Detection of hereditary angioneurotic edema by demonstration of a reduction in the second component of human complement. *N. Engl. J. Med.* **272**: 649.
- Kagan, L. J., and E. L. Becker. 1963. Inhibition of permeability globulins by C'1 esterase inhibitor. *Fed. Proc.* **22**: 613. (Abstr.)
- Kagan, L. J. 1964. Some biochemical and physical properties of human permeability globulins. *Brit. J. Exp. Pathol.* **45**: 604.
- Ratnoff, O. D., J. Pensky, D. Ogston, and G. B. Naff. 1969. The inhibition of plasmin, plasma kallikrein, plasma permeability factor (PF/Dil) and the C'1r subcomponent of the first component of complement by serum C'1 esterase inhibitor. *J. Exp. Med.* **129**: 315.
- Pensky, J., L. R. Levy, and I. H. Lepow. 1961. Partial purification of a serum inhibitor of C'1 esterase. *J. Biol. Chem.* **236**: 1674.
- Miles, A. A., and D. L. Wilhelm. 1960. 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. Exp. Pathol.* **36**: 71.
- de Jalon, P. G., Y. M. Bayo Bayo, and M. G. de Jalon. 1945. Sensible y nuevo metodo de valoración de adrendina en utero osseaus de Rata. *Farmacoter.* **2**: 313.
- Erdös, E. G., V. Jackman, and W. C. Barnes. 1962. Instrument for recording isotonic contractions of smooth muscles. *J. Appl. Physiol.* **17**: 367.
- Laurell, C. B., S. Laurell, and N. Skoog. 1956. Buffer composition in paper electrophoresis. Considerations on its influence with special reference to the interaction between small ions and proteins. *Clin. Chem.* **2**: 99.
- Oates, J. A., W. A. Pettinger, and R. B. Doctor. 1966. Evidence for the release of bradykinin in carcinoid syndrome. *J. Clin. Invest.* **45**: 173.
- Rocha e Silva, M., W. T. Beraldo, and G. Rosenfeld. 1949. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Amer. J. Physiol.* **156**: 261.
- Erdös, E. G., J. R. Wohler, and M. I. Levine. 1963. Blocking of the in vivo effects of bradykinin and kallidin with carboxypeptidase B. *J. Pharm. Exp. Ther.* **142**: 327.
- Webster, M. E., and J. P. Gilmore. 1965. The estimation of the kallidins in blood and urine. *Biochem. Pharmacol.* **14**: 1161.
- Dias da Silva, W., and I. H. Lepow. 1966. Anaphylatoxin formation by purified human C'1 esterase. *J. Immunol.* **95**: 1080.
- Rothschild, A. M., and M. Rocha e Silva. 1954. Activation of a histamine-releasing agent (anaphylatoxin) in normal rat plasma. *Brit. J. Exp. Pathol.* **35**: 507.
- Dias da Silva, W., and I. H. Lepow. 1967. Complement as a mediator of inflammation. II. Biological properties of anaphylatoxin prepared with purified elements of human complement. *J. Exp. Med.* **125**: 921.
- Jensen, J. 1967. Anaphylatoxin and its relation to the complement system. *Science.* **155**: 1122.
- Dias da Silva, W., J. W. Eisele, and I. H. Lepow. 1967. 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. *J. Exp. Med.* **126**: 1027.
- Cochrane, C. G., and H. J. Müller-Eberhard. 1968. The derivation of two distinct anaphylatoxin activities from the third and fifth components of human complement. *J. Exp. Med.* **127**: 371.
- Erdös, E. G., and E. M. Sloane. 1962. An enzyme in human blood plasma that inactivates bradykinin and kallidins. *Biochem. Pharmacol.* **11**: 585.
- Bishop, E., and J. Margolis. 1963. Studies on plasma kinins. II. Some properties of the kinin-destroying enzyme. *Aust. J. Exp. Biol. Med. Sci.* **41**: 307.
- Donaldson, V. H. 1968. C'1 activation in hereditary angioneurotic edema plasma: role of urokinase and inhibitors. *J. Appl. Physiol.* **25**: 416.
- Ratnoff, O. D., and A. A. Miles. 1964. The induction of permeability-increasing activity in human plasma by

- activated Hageman factor. *Brit. J. Exp. Pathol.* **45**: 328.
28. Donaldson, V. H. 1968. Mechanisms of activation of C'1 esterase in hereditary angioneurotic edema plasma in vitro. The role of Hageman factor, a clot-promoting agent. *J. Exp. Med.* **127**: 411.
 29. Taylor, F. B., Jr., and H. Fudenberg. 1964. Inhibition of the C'1 component of complement by amino acids. *Immunology.* **7**: 319.
 30. Ablondi, F. B., J. J. Hagan, M. Philips, and E. C. DeRenzo. 1959. Inhibition of plasmin, trypsin, and the streptokinase-activated fibrinolytic system by 6-aminocaproic acid. *Arch. Biochem. Biophys.* **82**: 153.
 31. Alkjaersig, N., A. P. Fletcher, and S. Sherry. 1959. Epsilon aminocaproic acid: an inhibitor of plasminogen activation. *J. Biol. Chem.* **234**: 832.
 32. Armstrong, D., and J. W. Stewart. 1960. Formation of plasma kinin in human plasma by epsilon aminocaproic acid and certain related compounds. *J. Physiol.* **154**: 19P. (Abstr.)
 33. Kettel, L. J., F. J. Haddy, and D. P. Earle. 1966. Forelimb vascular responses to infusions of EACA in dogs. *Clin. Res.* **14**: 252.
 34. Lundh, B., A. B. Laurell, H. Wetterquist, T. White, and G. Granerus. 1968. A case of hereditary angioneurotic edema treated with epsilon aminocaproic acid. *Clin. Exp. Immunol.* **3**: 733.
 35. Webster, M. E., and J. V. Pierce. 1961. Action of the kallikreins on synthetic ester substrates. *Proc. Soc. Exp. Biol. Med.* **107**: 186.
 36. Mirsky, I. A. 1944. Inhibition of beta hemolytic streptococcal fibrinolysin by trypsin inhibitor (antiprotease). *Science.* **100**: 198.
 37. Breckenridge, R. T., and Ratnoff, O. D. 1965. The role of proaccelerin in human blood coagulation. Evidence that proaccelerin is converted to a prothrombin-converting principle by activated Stuart factor: with notes on the anticoagulant action of soybean trypsin inhibitor, protamine sulfate, and hexadimethrine bromide. *J. Clin. Invest.* **44**: 302.
 38. Elder, J. M., and D. L. Wilhelm. 1958. Enzyme-like globulins from serum reproducing the vascular phenomena of inflammation. V. Activable permeability factor in human serum. *Brit. J. Exp. Pathol.* **39**: 23.
 39. Ratnoff, O. D., and I. H. Lepow. 1957. Some properties of an esterase derived from preparations of the first component of complement. *J. Exp. Med.* **106**: 327.
 40. Kraut, H., and N. Bhargava. 1964. Versuche zur Isolierung des Kallikrein-Inaktivators. V. Isolierung eines Kallikrein-Inaktivators aus Rinderlunge und seine Identifizierung mit dem Inaktivator aus Rinderparotis. *Z. Physiol. Chem.* **338**: 231.
 41. Anderer, F. A., and S. Z. Hörnle. 1965. Strukturuntersuchungen am Kallikrein-Inaktivator aus Rinderlunge. I. Molekulargewicht, Endgruppenanalyse und Aminosäure-Zusammensetzung. *Z. Naturforsch. Pt. B.* **20**: 457.
 42. Klemperer, M. R., V. H. Donaldson, and F. S. Rosen. 1968. Effect of C'1 esterase on vascular permeability in man: studies in normal and complement-deficient individuals and in patients with hereditary angioneurotic edema. *J. Clin. Invest.* **47**: 604.
 43. Mayer, M. M., E. T. Asher, and T. Borsos. 1962. Inhibition of guinea pig C'2 by rabbit anti-C'2. *Fed. Proc.* **21**: 17. (Abstr.)
 44. Sitomer, G., R. M. Stroud, and M. M. Mayer. 1966. Reversible adsorption of C'2 by EAC'4: role of Mg²⁺, enumeration of competent SAC'4, two-step nature of C'2 fixation and estimation of its efficiency. *Immunochemistry.* **3**: 57.
 45. Eisen, V. 1963. Observations on intrinsic kinin-forming factors in human plasma: the effect of acid, acetone, chloroform, heat and euglobulin separation on kinin formation. *J. Physiol.* **166**: 496.
 46. Armstrong, D. A., and G. L. Mills. 1964. A highly purified kinin-forming enzyme from human plasma. *Biochem. Pharmacol.* **13**: 1393.
 47. Landerman, N. S., M. E. Webster, E. L. Becker, and H. E. Ratcliffe. 1962. Hereditary angioneurotic edema. II. Deficiency of inhibitor for serum globulin permeability factor and/or plasma kallikrein. *J. Allergy.* **33**: 330.
 48. Burdon, K. L., J. T. Queng, O. C. Thomas, and J. P. McGovern. 1965. Observations on biochemical abnormalities in hereditary angioneurotic edema. *J. Allergy.* **36**: 546.