INCREASED VASCULAR PERMEABILITY INDUCED BY HUMAN PLASMIN*

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Under suitable conditions, polypeptides which can evoke elements of the inflammatory reaction appear in mammalian plasma or inflammatory exudates. These polypeptides, the plasma kinins, increase vascular permeability, contract smooth muscle, dilate blood vessels, initiate leukocytic infiltration, and provoke pain (1). The kinins are derived from a protein precursor, prokinin or kininogen.

Several groups of investigators have pointed out that plasmin, the proteolytic enzyme or enzymes of plasma which can digest many substrates at neutrality, can induce the formation of kinins, either directly or through an action upon kallikreinogen (2–8). The presence of kinins was recognized by their ability to contract smooth muscles or dilate blood vessels. Curiously, it has not been possible to demonstrate that plasmin increases vascular permeability, one of the properties attributed to kinins (9–11). The reasons for this discrepancy are not clear.

In the present study, the effect of plasmin upon vascular permeability has been reexamined. Partially purified plasminogen was prepared in such a way that it was soluble in aqueous media at neutrality, and was relatively deficient in Hageman factor. Plasmin derived from this preparation regularly increased vascular permeability in guinea pig skin, an effect inhibited by soybean trypsin inhibitor. When plasmin was incubated with fractions of plasma rich in prokinins, permeability-enhancing properties appeared in the mixture. These properties could not be blocked by soybean trypsin inhibitor or heparin, suggesting that they might be due to the presence of kinins. The possibility must be entertained that plasmin may contribute to the increase in vascular permeability characteristic of the acute inflammatory reaction.

Materials

Partially purified plasminogen was prepared by adaptations of methods of Robbins and Summaria (12), Cole (13), and Hagan (14) from Cohn fraction III_4 of human plasma.[1] Cohn

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[1] Cohn Fraction III_4 was furnished by the Cutter Laboratories, Berkeley, California.

fraction III_4 was chromatographed successively upon columns of hydroxylapatite, carboxymethylcellulose, and diethylaminoethyl sephadex, each column being 24 cm high and 2.7 cm in diameter. The protein content of fractions of the eluate was estimated approximately by measuring their ultraviolet absorption at a wave length of 280 μ with a Gilson medical electronics ultraviolet absorption meter.

In a typical preparation, 8 gm of fraction III_4, containing 15 per cent protein, was dissolved in a solution of 0.2 M sodium chloride and 0.02 M L-lysine monohydrochloride (Nutritional Biochemicals Co., Cleveland) at pH 7.0 and dialyzed overnight against 3 liters of this buffer. The fraction was then applied to a column of hydroxylapatite (hypatite C, Clarkson Chemical Co., Inc., Williamsport, Pennsylvania) previously equilibrated with the same buffer. The plasminogen was eluted with a linear gradient of 250 ml each of this buffer and of 0.4 M potassium phosphate (pH 6.8) containing 0.02 M L-lysine monohydrochloride. Material containing plasminogen was eluted after approximately 140 ml of buffer had entered the column, coincident with the appearance of an asymmetric peak of protein.

The 230 ml of eluate with maximal plasminogen activity was dialyzed for 8 hours against 3 liters of 0.01 M sodium acetate (pH 5.2) and applied to a column of carboxymethylcellulose (15) equilibrated with this buffer. Contaminating protein was eluted with a linear gradient of 125 ml each of 0.01 M sodium acetate (pH 5.2) and 0.01 M sodium acetate (pH 5.2) in 0.1 M sodium chloride; both buffers contained 0.02 M L-lysine monohydrochloride. Fractions containing plasminogen were eluted with a linear gradient of 150 ml of the sodium acetate-sodium chloride-lysine buffer and 150 ml of 0.05 M trishydroxymethylamino-methane (tris), 0.1 M sodium chloride, and 0.02 M L-lysine hydrochloride at pH 9.0. Material containing plasminogen was eluted after 200 ml of the gradient had entered the column. An additional 100 ml of the final buffer was applied to complete elution. At least three components could be identified in the fractions containing plasminogen as measured by ultraviolet absorption during chromatography.

The active fractions were pooled, dialyzed for 2 hours against the tris-lysine-sodium chloride buffer and applied to a column of diethylaminoethyl sephadex A-50 (Pharmacia Fine Chemicals, New York). Plasminogen did not adhere to the column and was collected by elution with the same buffer. The fractions containing plasminogen were pooled and stored at 4°C. After dialysis against 0.15 M sodium chloride solution, the plasminogen could be lyophilized without loss. The solutions of plasminogen were by no means pure, containing approximately 75 to 120 Remmert and Cohn units per mg of nitrogen, as determined by Kjeldahl analysis.

In different batches, approximately 10 to 30 per cent of the "plasminogen" was in the form of plasmin, measured by the capacity to digest casein without further activation. It contained trace amounts of clot-promoting materials which shortened the abnormally long clotting time of plasma deficient in Hageman factor, as tested by an assay described earlier (16). Assuming that the clot-promoting activity was due to Hageman factor, there were approximately 0.0005 units of Hageman factor per unit of plasminogen, one unit of Hageman factor being that amount found in one ml of pooled normal human plasma (16). Trace amounts of activity assayed as plasma thromboplastin antecedent (PTA, factor XI), Christmas factor (factor IX), factor VII, and Stuart factor (factor X) were present, but there was no measurable antihemophilic factor (factor VIII), proaccelerin (factor V), nor prothrombin (factor II), (16, 17). No activity resembling C'1 esterase or C'1 subcomponents was detectible.

Before injection into guinea pig skin, the solution of plasminogen was dialyzed overnight against barbital-saline buffer containing 0.02 M lysine.

Crude preparations containing *prokinin* were separated from human plasma by two methods. In one, the fraction soluble at 4°C at 30 per cent saturation but insoluble at half-saturation with neutral ammonium sulfate was precipitated from citrated plasma deficient in

Hageman factor. The precipitate, dissolved in its original volume of water, was dialyzed overnight against barbital-saline buffer and then diluted with one-tenth volume of 5 per cent sodium ethylenediaminetetraacetic acid (pH 7.0). Half of the globulin was incubated for 30 minutes in cellulose nitrate tubes in a water-bath set at 62°C. This procedure is said to deplete plasma of kallikreinogen (18, 19) and the precursor of human plasma PF/Dil (20, 21) but to leave prokinin intact (19, 22).

The method just described is subject to the objection that the preparation was heated. Prokinin was therefore separated in a second way by a minor adaptation of the technique of Webster and Pierce (23). One hundred ml of citrated normal human plasma, separated from out-dated bank blood, was diluted to 400 ml with water and the pH adjusted to 6.0 with 1 M acetic acid. The diluted plasma was stirred with 4 gm of diethylaminoethyl (DEAE)-cellulose (selectacel Type 20, capacity 1.17 meq per gm, Carl Schleicher and Schuell Co., Keene, New Hampshire) which had been equilibrated with 0.005 M potassium phosphate (pH 6.0). The filter cake was then eluted three times with 50 ml each of 0.2 M potassium phosphate (pH 6.0), centrifuging each time at 3500 RPM for 10 minutes. The combined eluates were recentrifuged, the supernatant solution dialyzed overnight against 0.005 M potassium phosphate (pH 6.0), and the precipitate which formed was removed by centrifugation. The supernatant fluid was applied to a DEAE-cellulose column, 22 cm in height and 2.7 cm in diameter, previously equilibrated with the same buffer. The column was eluted successively by 100 ml 0.005 M and 300 ml of 0.08 M potassium phosphate (pH 6.0), and then by a linear gradient of 250 ml each of 0.08 and 0.2 M potassium phosphate (pH 6.0). Material containing prokinin-like activity was eluted after approximately 200 ml of the gradient mixture had entered the column. Maximal activity, in fractions totalling 125 ml, coincided with the central portion of an almost symmetrical peak of protein, as measured by ultraviolet absorption. Although kallikreinogen is said not to adhere to DEAE-cellulose (24), proof that this material was not contaminated with kallikreinogen was lacking.

Streptokinase, "high purity,[2] dissolved in buffer at concentrations of 1000 or 2000 Christensen units per ml, or purified human *urokinase*,[3] dissolved at various concentrations, were used to change plasminogen to plasmin. The preparation of urokinase used contained 275,000 "C.T.A." units per mg of nitrogen.

Soybean trypsin inhibitor (SBTI), 5 times crystallized, (Nutritional Biochemicals Corp., Cleveland), was suspended in buffer at a concentration of 2 mg per ml, and diluted suitably as needed.

Epsilon aminocaproic acid (Mann Research Lab., Inc., New York) was dissolved at a concentration of 0.8 M in water.

Heparin[4] (156 Toronto units per mg) was dissolved in buffer.

Triprolidine hydrochloride (actidil),[5] an antihistaminic agent, was dissolved at a concentration of 1.0 mg (calculated as free base) per ml of 0.15 M sodium chloride solution.

Histamine diphosphate (U.S.P., *B* grade, Calbiochem, Los Angeles) was dissolved at a concentration of 55 μg per ml of buffer, corresponding to 20 μg of free base per ml.

Sodium ethylenediaminetetraacetic acid (EDTA) was dissolved at a concentration of 5 per cent in water and brought to pH 7.4 by the addition of sodium hydroxide.

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[2] Streptokinase was the gift of the American Cyanamid Company, Lederle Laboratories Division, Pearl River, New York.

[3] Urokinase was provided by the Sterling-Winthrop Research Institute, Rensselaer, New York.

[4] Purified heparin was furnished by the Upjohn Company, Kalamazoo, Michigan.

[5] Triprolidine hydrochloride was the gift of Burroughs, Wellcome and Company, Inc., Tuckahoe, New York.

Hageman factor, partially purified by a method described before (16), was dissolved in buffer at a concentration of 4 or 8 μg protein per ml. A single lot, "December 4, 1961", containing 8 per cent protein and 52 units of Hageman factor per mg of protein was used. Only one per cent of the Hageman factor in this preparation was activated, the rest being in the form of an inert precursor.

Crystalline bovine *trypsin*, twice crystallized (Worthington Biochemical Corp., Freehold, New Jersey), was dissolved in buffer just before use. The preparation contained approximately 50 per cent magnesium sulfate. The concentrations of trypsin recorded are uncorrected for this contaminant.

Hageman factor-deficient plasma was prepared from blood obtained from a patient with Hageman trait, to which one-ninth volume of 0.13 M sodium citrate buffer (pH 5.0) had been added.

All *dialyses* were performed at 4°C in visking no-jax cellophane casings. All *centrifugations* were at 2°C.

Unless otherwise specified, the *buffer* used was 0.025 M barbital in 0.125 M sodium chloride solution at pH 7.5.

Methods

Vascular permeability was tested by the method of Miles and Wilhelm (25) in albino guinea pigs of either six which usually weighed between 450 and 500 gm. The guinea pigs were depilated and injected intravenously with 1.2 ml per kg of body weight of a 5 per cent solution of pontamine sky blue 6 X (E. I. Dupont de Nemours and Co., Wilmington) in 0.075 M pyrogen-free sodium chloride solution. Beginning within the succeeding 10 minutes, unless otherwise noted, the solutions to be tested were injected intracutaneously into the dorsum of the guinea pigs in a volume of 0.1 ml, using No. 26 gauge disposable needles (Becton, Dickinson and Co., Rutherford, New Jersey) and silicone-coated tuberculin syringes. 15 minutes after the last injection, the size of the lesion was estimated as the average of the widest diameter and the diameter perpendicular to it. Permeability-enhancing activity was expressed in terms of the average diameter of the lesions in three or four guinea pigs. An occasional guinea pig reacted with an increase in vascular permeability to any solution containing human plasma proteins. The values obtained in such "sensitive" guinea pigs were not included in the data presented. The increase in vascular permeability which resulted from an injection of buffer was significantly less when disposable needles were used than with stainless steel needles, used in earlier studies.

An effective blueing dose of material is that amount in a volume of 0.1 ml which produces a lesion whose average diameter, 15 minutes after injection, is 6.0 mm (25).

The effect of plasmin upon vascular permeability was tested by activating plasminogen with streptokinase or urokinase. In general, equal volumes of plasminogen solution and solutions of streptokinase or urokinase were incubated at room temperature in cellulose nitrate tubes for 15 minutes and then stored in ice for as long as 45 minutes until injected. *The inhibitory effect of SBTI upon the activity of plasmin* was tested by incubating one volume of plasminogen with one-half volume of streptokinase or urokinase for 15 minutes, and then adding one-half volume of SBTI or buffer. The mixtures were stored in ice until injected. *The inhibitory effect of epsilon aminocaproic acid upon the activation of plasmin* was measured by incubating one volume of plasminogen, one-half volume of streptokinase or urokinase, and one-half volume of epsilon aminocaproic acid or buffer together at room temperature for 15 minutes and then storing the mixtures in ice.

The effect of triprolidine hydrochloride upon the permeability-enhancing properties of plasmin was tested by comparing the effect of the intravenous injection of 0.1 ml per kg of body weight of this agent, at a concentration of 1 mg free base per ml, with 0.15 M sodium

chloride solution. The triprolidine or sodium chloride solutions were administered with the pontamine sky blue dye, before the intracutaneous injection of test substances. The effectiveness of the injection of triprolidine was tested by the intracutaneous injection of histamine.

The effect of plasmin and other enzymes upon crude prokinin, prepared by the method of Webster and Pierce (23), was studied by incubating a mixture of 0.18 ml of "prokinin" solution, 0.02 ml of 5 per cent EDTA solution, 0.1 ml of the enzyme to be tested and 0.1 ml of buffer in silicone-coated Pyrex tubes (8 mm internal diameter) at 25°C for 30 minutes. The mixture was then transferred to an ice-bath and diluted to 2.0 ml with buffer before injection. The enzymes tested were plasmin, prepared by mixing an equal volume of plasminogen (2.5 units per ml) and streptokinase (2000 units per ml), trypsin, 0.1 mg per ml and Hageman factor, 8μg of protein per ml, the concentrations referring to the solutions before their addition to prokinin. *The effect of SBTI and heparin upon the action of these enzymes* was tested by substituting these inhibitors for buffer in both the initial mixture and the subsequent diluting fluid. The concentration of SBTI was maintained at 250/ μg per ml, and of heparin, at 600 units per ml throughout the experiment. *The effect of SBTI and heparin upon the product formed* from the reaction between the enzymes and prokinin was tested by diluting the prokinin-enzyme mixtures with these inhibitors after the initial 30 minute incubation period. The final concentration of SBTI was 250 μg per ml, and of heparin, 600 units per ml.

The effect of plasmin and other enzymes upon crude prokinin in a globulin fraction of Hageman factor–deficient plasma was tested by a similar technique. A mixture of 0.2 ml of globulin, either unheated or heated at 62°C for 30 minutes, and 0.2 ml of enzyme or buffer was incubated in silicone-coated cellulose nitrate tubes (internal diameter 15 mm) at 25°C for 30 minutes, transferred to an ice-bath and diluted to 2.0 ml with buffer before injection. The enzymes tested were trypsin, 0.1 mg per ml, Hageman factor 4 μg of protein per ml and plasmin, prepared by mixing an equal volume of plasminogen (3.5 units per ml) and streptokinase (2000 units per ml), the concentrations referring to the solutions before their addition to globulin. The globulin solution contained one-tenth volume of 5 per cent EDTA.

In experiments with prokinin, EDTA was included in the incubation mixtures to inhibit any kininase which might have been present in the preparations, lest this enzyme inactivate kinins which might form (26, 27).

The Concentration of Plasminogen was measured by the method of Remmert and Cohen (28), as modified by Kline (29). One unit of plasminogen was that amount which, under the conditions of this assay, released 450g μg of tyrosine-like material from a substrate of 1.5 per cent casein in 1 hour.

RESULTS

The Permeability-Enhancing Activity of Plasmin.—The preparations of plasminogen tested invariably contained some active plasmin, as measured by their ability to digest casein. Given in sufficient concentration, the intracutaneous injection of such preparations increased vascular permeability (Table I). When plasminogen solutions were mixed with streptokinase or urokinase, the permeability-enhancing activity was greatly increased. An "effective blueing dose" of plasmin was approximately 0.1 unit of caseinolytic activity. In control experiments, streptokinase, at a concentration of 500 units per ml, had no effect upon vascular permeability. Urokinase, at a concentration of 4000 units per ml, slightly increased vascular permeability, while 1000 units per ml was without effect.

The caseinolytic and fibrinolytic properties of plasmin can be inhibited by

soybean trypsin inhibitor. Similarly, the permeability-increasing property of spontaneously active plasmin, or plasmin activated by streptokinase or urokinase was regularly inhibited by SBTI at a concentration of 125 to 250 μg per ml (Table II).

TABLE I

Permeability-Enhancing Activity of Plasminogen, Plasmin, Streptokinase, and Urokinase

Mixture tested*	Permeability activity‡
	mm
Plasminogen, 2.5 units per ml	5.7
Plasminogen, 2.5 units per ml, in streptokinase, 500 units per ml	9.2
Plasminogen, 0.83 units per ml, in streptokinase, 500 units per ml	6.0
Plasminogen, 2.5 units per ml, in urokinase, 1000 units per ml	8.7
Plasminogen, 0.83 units per ml, in urokinase, 1000 units per ml	5.3
Streptokinase, 500 units per ml	2.3
Urokinase, 4000 units per ml	4.0
Urokinase, 2000 units per ml	3.8
Urokinase, 1000 units per ml	2.8
Buffer	2.5

* See Methods. The plasminogen preparation contained 30 per cent plasmin and 70 per cent plasminogen, as determined by caseinolysis.

‡ Diameter of lesion.

TABLE II

Inhibition of the Permeability-Enhancing Activity of Plasmin by Soybean Trypsin Inhibitor

Mixture tested*	Permeability activity‡
	mm
Plasminogen, 2.5 units per ml in streptokinase, 500 units per ml	8.6
Plasminogen, 2.5 units per ml in streptokinase, 500 units per ml and SBTI, 125 μg per ml	3.9
Plasminogen, 2.5 units per ml in urokinase, 1000 units per ml	8.7
Plasminogen, 2.5 units per ml in urokinase, 1000 units per ml and SBTI, 250 μg per ml	1.5
Plasminogen, 2.5 units per ml in urokinase, 1000 units per ml and SBTI, 125 μg per ml	5.7

* See Methods.

‡ Diameter of lesion.

The change induced in the skin by plasmin was transient. Within 11 minutes after the intracutaneous injection of streptokinase-activated plasmin, its local effect was drastically diminished. After 41 minutes, increased vascular permeability was no longer demonstrable in the injected areas (Table III).

The activation of plasminogen by streptokinase or urokinase can be inhibited by epsilon aminocaproic acid. When plasminogen was incubated with streptokinase in the presence of 0.2 M epsilon aminocaproic acid, the mixture had two-fifths the permeability-increasing activity of a similar mixture from which the epsilon aminocaproic acid had been omitted, as estimated by interpolation (Table IV). Complete inhibition was not to be expected, since the plasminogen solution contained spontaneously active plasmin, only poorly inhibited by epsilon aminocaproic acid at the concentration tested. The permeability-increasing property of plasmin was not inhibited by two intraperitoneal injections of epsilon aminocaproic acid, in a dose of 1 gm per kilo of body weight, 45 and 15 minutes before.

TABLE III

The Duration of Increased Vascular Permeability After the Injection of Plasmin

Age of lesion when dye injected*	Permeability activity‡
minutes	*mm*
0	8.3
6	8.8
11	4.7
21	3.7
41	2.3

* A mixture of plasminogen, 2 units per ml, in streptokinase, 500 units per ml, was incubated at 25°C in cellulose nitrate tubes for 15 minutes and then stored in ice. At intervals, 0.1 ml aliquots were injected into guinea pigs before and just after the intravenous injection of pontamine sky blue.

‡ Diameter of lesion.

Experiments on the Mode of Action of Plasmin in Increasing Vascular Permeability.—In earlier studies, the suggestion was made that plasmin can change the first component of complement to a hydrolytic enzyme, C'1 esterase (30). C'1 esterase increases vascular permeability in guinea pig skin, an effect inhibited by the intravenous injection of triprolidine (31). In contrast, triprolidine did not inhibit the permeability-enhancing activity of plasmin (Table V).

Preparations of partially purified activated Hageman factor increase vascular permeability (21). One explanation for the effect of plasmin might be its contamination with activated Hageman factor. The effective blueing dose of preparations of Hageman factor used in previous experiments was approximately 0.02 to 0.04 units per 0.1 ml (21). In contrast, the amount of Hageman

factor contaminating the plasminogen preparations was approximately 0.00005 units per 0.1 ml. It seems unlikely, therefore, that the effect of plasmin could be attributed to its content of this clotting factor.

A more sensitive test for contamination of the plasminogen with activated

TABLE IV

The Effect of Epsilon Aminocaproic Acid Upon the Permeability-Enhancing Properties of Plasmin

Mixture tested*	Permeability activity‡
	mm
Plasminogen, 1.6 units per ml, in streptokinase, 500 units per ml	7.5
Plasminogen, 0.55 units per ml, in streptokinase, 500 units per ml	4.0
Plasminogen, 1.6 units per ml, in buffer	4.0
Plasminogen, 1.6 units per ml, in streptokinase, 500 units per ml and epsilon aminocaproic acid, 0.2 M	5.0
Buffer	2.5

* See Methods. The plasminogen preparation contained approximately 15 per cent plasmin and 85 per cent plasminogen, as determined by caseinolysis.

‡ Diameter of lesion.

TABLE V

The Effect of Intravenously Injected Triprolidine Upon the Vascular Permeability-Enhancing Properties of Plasmin

Mixture tested*	Permeability activity‡	
	Triprolidine	Buffer
	mm	*mm*
Histamine diphosphate, 55 μg per ml	3.0	9.2
Plasminogen, 2 units per ml, in streptokinase, 500 units per ml	9.8	8.8
Plasminogen, 2 units per ml, in urokinase, 1000 units per ml	8.8	9.2
Buffer	2.7	2.7

* See Methods.

‡ Diameter of lesion.

Hageman factor took advantage of the observation that this clotting factor can activate the permeability factor in human plasma designed by Miles and Wilhelm as PF/Dil (25). An amount of plasminogen was chosen which of itself was insufficient to produce a measurable increase in vascular permeability without the addition of streptokinase. This amount of plasminogen, 1 unit per

ml, was incubated for 24 minutes at 37°C with an equal volume of Hageman factor–deficient plasma, diluted 50-fold with buffer in silicone-coated cellulose nitrate tubes. No permeability-enhancing activity evolved, as tested 0, 12, and 24 minutes after the mixtures were prepared. In contrast, a mixture containing 1 μg of partially activated Hageman factor in 100-fold diluted Hageman factor–deficient plasma had sufficient permeability-enhancing activity to produce an area of blueing 8.1 mm in diameter in 12 minutes. Presumably, then, the

TABLE VI

The Effect of Trypsin, Hageman Factor, and Plasmin Upon the Permeability-Enhancing Properties of Crude "Prokinin": Influence of Inhibitors

Agents incubated with "prokinin" and enzyme*	Diluent added after incubation*	Permeability activity‡			
		Trypsin	Hageman factor	Plasmin	Buffer
		mm	*mm*	*mm*	*mm*
Buffer	Buffer	8.6	7.0	7.9	3.4
SBTI, 250 μg per ml§	SBTI, 250 μg per ml‖	3.0	2.8	3.4	
Buffer	SBTI, 250 μg per ml‖	8.0	3.0	8.0	
Heparin, 600 units per ml§	Heparin, 600 units per ml‖	4.0	3.6	3.5	
Buffer	Heparin, 600 units per ml‖	8.1	4.0	7.0	

* A mixture of 0.18 ml of "prokinin", 0.02 ml 5 per cent EDTA, 0.1 ml of the enzyme to be tested and 0.1 ml of buffer, SBTI or heparin was incubated in silicone-coated tubes at 25°C for 30 minutes (See Methods). The mixtures were then diluted to 2.0 ml with buffer or solutions of SBTI or heparin, and injected into guinea pigs. The enzymes tested were trypsin, 0.1 mg per ml, Hageman factor, 8 μg protein per ml, and a mixture of plasminogen, 1.75 units per ml in streptokinase, 1000 units per ml. The concentrations of these enzymes refer to the solutions before their addition to prokinin.

‡ Diameter of lesion.

§ Concentration in the initial mixture.

‖ Concentration in the final mixture.

permeability-increasing property of plasmin could not be attributed to its contamination with activated Hageman factor.

Plasmin has been said to bring about the formation of plasma kinins, either by digestion of their precursor, prokinin, or by changing kallikreinogen to kallikrein. In this way, plasmin is apparently similar in behavior to trypsin.

When a fraction of human plasma thought to be rich in prokinin, prepared by a technique derived from that of Webster and Pierce, was incubated with trypsin, the mixture acquired the property of increasing vascular permeability (Table VI). This effect was prevented by SBTI or heparin, but these substances did not block the agents which evolved from the action of trypsin. In some preparations of crude prokinin, such as that used in the experiment summarized

in Table VI, permeability-increasing activity also evolved upon incubation with Hageman factor. Other preparations of prokinin made by the same method lacked this last property. The evolution of this activity was prevented by SBTI and heparin. Moreover, the agent which appeared in mixtures of prokinin and Hageman factor was inhibited by SBTI and heparin, distinguishing it from that which appeared in mixtures of prokinin and trypsin.

Permeability-increasing activity also appeared when the same preparations of prokinin were incubated with streptokinase-activated plasmin (Table VI). The development of this activity was inhibited by SBTI and heparin, but once it had evolved, these agents did not block its effect upon guinea pig skin. Thus the effect of plasmin was similar to that of trypsin, but unlike that of Hageman

TABLE VII

The Effect of Trypsin, Hageman Factor, and Plasmin Upon the Permeability-Enhancing Properties of a Globulin Fraction of Plasma

Fraction incubated with enzyme*	Permeability activity‡			
	Trypsin	Hageman factor	Plasmin	Buffer
	mm	*mm*	*mm*	*mm*
Unheated globulin	7.5	8.1	6.8	3.0
Globulin heated 62°C, 30 minutes	8.3	3.4	6.8	2.9
Buffer	2.9	3.0	3.6	2.5

* A mixture of 0.2 ml globulin or buffer was incubated at 25°C for 30 minutes in silicone-coated tubes with 0.2 ml enzyme or buffer, and then diluted to 2.0 ml with buffer, before injection (see Methods). The enzymes tested were trypsin 0.1 mg per ml, Hageman factor, 4 μg protein per ml, and a mixture of plasminogen, 2.5 units per ml in streptokinase, 1000 units per ml, the concentrations referring to the solutions before their addition to globulin.

‡ Diameter of lesion.

factor. In the concentrations tested, neither prokinin, plasminogen, nor streptokinase alone had a measurable effect.

The effect of plasmin upon a prokinin fraction of plasma could have been indirect, for it was not possible to exclude the presence of kallikreinogen. The following experiment suggests that plasmin can induce the formation of a permeability-increasing agent in plasma depleted of this proenzyme.

A globulin fraction was prepared from plasma deficient in Hageman factor and was incubated at 62°C for 30 minutes, a procedure designed to inactivate kallikreinogen. When this fraction was incubated with trypsin, the mixture acquired the capacity to increase vascular permeability (Table VII). Similarly, a mixture of streptokinase-activated plasmin and the heated globulin increased vascular permeability. No such activity evolved when partially activated Hageman factor was incubated with the heated globulin fraction. In contrast,

a mixture of Hageman factor and an unheated globulin fraction enhanced vascular permeability. These experiments demonstrate that plasmin can act upon an agent or agents not affected by activated Hageman factor. They do not prove that plasmin acts in the absence of kallikreinogen, since traces of this agent may have survived the heating process.

DISCUSSION

In the experiments reported here, human plasmin regularly increased vascular permeability in the skin of guinea pigs. Plasmin also induced the formation of permeability-increasing agents in a fraction of plasma containing the precursors of the kinins.

Under experimental conditions, a number of biologically active polypeptides may appear to be generated within human plasma. These polypeptides, the kinins, increase vascular permeability, dilate blood vessels, contract smooth muscles, induce pain, and provoke the migration of leukocytes into extravascular tissues (1). Three peptides have been identified, bradykinin (32), kallidin (33), and methionyl-lysylbradykinin (34), and still others may exist (35). The elaboration of kinins at the site of injury seems to explain some of the phenomena of the inflammatory reaction. In agreement with this view, kinins have been identified in experimental inflammatory exudates (36, 37) and in fluid obtained from inflamed joints (38, 39).

The kinins are thought to be derived from a precursor in the globulin fraction of plasma (40). They are apparently liberated when this precursor, prokinin, is partially hydrolyzed by the kallikreins, a group of one or several proteases found in saliva, snake venom (a specialized type of saliva), pancreatic juice, urine, and other secretions (1). Kinins can also be elaborated from their precursor through the action of trypsin (41) or of a specific proteolytic enzyme in plasma itself, plasma kallikrein (42). In freshly shed blood, kallikrein exists in an inactive form, kallikreinogen (42). The activation of plasma kallikreinogen can be brought about in several ways. For example, treatment of plasma with acetone (42) or trypsin (41) results in the activation of kallikreinogen. Thus, trypsin appears to liberate kinins both by a direct action upon prokinin and indirectly, through the formation of plasma kallikrein.

Kallikreinogen can also be activated by exposing plasma to glass surfaces. The effects of acetone and of glass appear to depend upon the presence in plasma of a clotting factor, Hageman factor (43, 44). This agent is present in freshly shed blood in an inert form, but it can be activated by contact with certain suitable insoluble substances (45, 46) or by the addition of solutions of ellagic acid (47). The effect of activated Hageman factor upon kallikreinogen may be indirect, for it has been found to activate still another hydrolytic enzyme, variously known as PF/Dil and plasma permeability factor, which can increase vascular permeability in guinea pig skin. Perhaps the sequence of events when

plasma comes in contact with glass is the successive activation of Hageman factor, PF/Dil and kallikreinogen, leading ultimately to the elaboration of the kinins.

As one might expect, the intracutaneous injection of the active form of any of the agents in this chain of reactions increases vascular permeability. Thus, the injection of activated Hageman factor, PF/Dil, kallikrein, or bradykinin augments vascular permeability. Under some conditions, these various permeability factors can be distinguished by suitable assays.

The mechanisms through which plasmin exerts its permeability-increasing effect was explored by techniques designed to distinguish its action from those of other known permeability factors. Plasminogen, the precursor of plasmin, had only such permeability-increasing activity as could be accounted for by the fraction of plasmin invariably present. Once plasminogen had been activated by streptokinase or urokinase, the plasmin which evolved enhanced vascular permeability. SBTI, which blocks the action of plasmin, inhibited its effect upon guinea pig skin. Epsilon aminocaproic acid, at the concentrations tested, is known to inhibit the activation of plasminogen, but has little effect upon plasmin. In agreement with this, this inhibitor was only partially effective in blocking the permeability-increasing properties of plasmin.

The experiments just summarized make it unlikely that the permeability-increasing properties of plasmin were due to contamination of the plasminogen preparation with other permeability factors. Among those described in human plasma are Hageman factor, PF/Dil, kallikrein, PF/Nat, PF/P, and C′1 esterase.

The preparation of plasminogen tested contained traces of Hageman factor, but these were far less than the amount needed to increase vascular permeability in guinea pig skin (21), and were insufficient to induce the formation of PF/Dil in diluted human plasma. Nor could the effect of streptokinase or urokinase upon the preparation of plasminogen be attributed to an action upon Hageman factor, since the activity of this procoagulant substance is not enhanced by these agents.

The fact that its activity was greatly enhanced by streptokinase and urokinase separated plasmin from PF/Dil, which is unaffected by these agents. That human plasmin is not PF/Dil has previously been shown by Mill and his colleagues (48). Nor can plasmin be identified with kallikrein (4, 9). Recently, McConnell, Kagen, and Becker (49) have shown that heparin, at a concentration of 600 units per ml, will not inhibit the permeability-increasing activity of kallikrein. In the present study, the effect of plasmin was completely blocked by preparations of heparin at this concentration. Whether the inhibitory effect was due to heparin itself or to some contaminant in the preparation was not examined.

The permeability-increasing activity of our preparations was clearly separa-

ble from that of PF/Nat, a permeability factor found in undiluted human plasma (20), because it was inhibited by SBTI and its effect lasted only a matter of minutes. It was distinguishable from PF/P which is elicited by exposure of guinea pig serum to antigen-antibody aggregates, since the latter is partially inhibited by the intravenous injection of antihistaminic agents, and, unlike plasmin, has a prolonged action after its injection into guinea pig skin (50).

Still another permeability factor which can be derived from human plasma is C′1 esterase, a hydrolytic enzyme which evolves during the activation of the first component of complement. The permeability-increasing properties of C′1 esterase are inhibited by the intravenous injection of triprolidine, an antihistaminic agent (31). Failure of triprolidine to block the action of plasmin distinguished its effect from that of C′1 esterase and made it unlikely that its action was mediated through the activation of the first component of complement. This observation is particularly pertinent, since evidence exists that plasmin can convert the first component of complement to C′1 esterase (30).

The possibility that plasmin exerted its effect by liberating kinins from their precursor, prokinin, was explored. The same amount of permeability-increasing activity evolved when plasmin was incubated with an unheated globulin fraction of plasma, as with one which had been heated at 62°C for 30 minutes, a procedure designed to decrease its content of kallikreinogen (18) and the precursor of PF/Dil (20, 21). Were kallikrein required for this action of plasmin, one might expect the heated globulin to produce a smaller lesion. Permeability-enhancing activity also appeared when plasmin was incubated with a fraction of plasma rich in prokinin which was prepared by column chromatography. The permeability factors which developed under these conditions were not inhibited by SBTI, distinguishing them from kallikrein (18, 51) or PF/Dil (20, 25), but not from non-enzymatic molecules like bradykinin (9).

The experiments described are in agreement with previous reports that plasmin (5, 7), like trypsin (41), can induce kinin-like activity by a direct action upon prokinin. They do not exclude an additional effect upon kallikreinogen, with which our prokinin preparations may have been unwittingly contaminated. Back (52) and Vogt (8) have described experiments which they interpret to mean that plasmin can convert kallikreinogen to kallikrein. No direct test of this possibility was made.

The observation that plasmin enhances vascular permeability is by no means surprising, since this enzyme has been shown to bring about the formation of agents which contract smooth muscle and dilate blood vessels (2–8). What is surprising is that earlier investigators failed to note the effect of plasmin upon vascular permeability. A likely explanation is that the preparations previously tested consisted principally of streptokinase and had little plasmin-like activity.

The degree to which plasmin may participate in the inflammatory reaction is not clarified by the present experiments. The concentration of plasmin which

was capable of increasing vascular permeability was approximately 1 Remmert and Cohen unit per ml. This is considerably less that the concentration of plasmin which might be generated in plasma. But plasma contains several inhibitory substances which can block its activity. Whether sufficient plasmin may become activated in areas of inflammation to a degree sufficient to overcome these inhibitors, and thus to contribute to the pathologic changes observed remains to be determined.

SUMMARY

Preparations of plasminogen, soluble in aqueous media, increased vascular permeability in guinea pig skin when activated by streptokinase or urokinase. The permeability-enhancing effect was inhibited by soybean trypsin inhibitor but not by triprolidine. Permeability-enhancing activity evolved when plasmin was incubated with fractions of plasma rich in prokinin. The experiments described suggest that plasmin exerts its permeability-increasing effect through the elaboration of kinins.

The experiments described were made possible by the expert technical assistance of Miss Gloria Bauer, Miss Linda Graham, Mrs. Marjorie Smink, and Mrs. Edna Stone. Dr. Arnold Powell determined the nitrogen content of our plasminogen preparations. Dr. I. H. Lepow assayed the plasminogen preparation for C′1 esterase and C′1 subcomponents.

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