PATHOGENESIS OF INFLAMMATION

II. In Vivo Observations of the Inflammatory Effects of Activated Hageman Factor and Bradykinin*

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Plates 60 to 62

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When exposed to glass, mammalian plasma acquires the ability to accelerate clotting, increase vascular permeability, induce pain, cause contraction of smooth muscle, and dilate blood vessels. These properties are dependent upon the presence of Hageman factor, a plasma protein which circulates in inactive form until "activated" by contact with glass or a number of other insoluble, negatively charged substances (1). Recently it has been demonstrated that very dilute solutions of ellagic acid also can activate Hageman factor by a mechanism as yet unknown (2). The activated Hageman factor then activates plasma thromboplastin antecedent (PTA), thus initiating the series of reactions which lead to clotting (3).

Hageman factor, activated by ellagic acid, has been shown to increase vascular permeability when injected into the skin of the guinea pig (4). Because the relationship of increased vascular permeability (as demonstrated by the extravasation of protein-bound dye) to the margination and emigration of leucocytes is still unclear, it seemed desirable to test the effects of activated Hageman factor in a system permitting the direct visualization of the microcirculation. In the present studies this has been done, utilizing a modification of the Sandison-Clark rabbit ear chamber. It also seemed of interest to compare the inflammatory effects of Hageman factor with those of bradykinin, since the activation of Hageman factor has been shown to be associated with the appearance of kinin-like activity in plasma (5).

Methods and Materials

Rabbit Ear Chamber.—The ear chamber used in these experiments has been described in detail in the previous communication (6). It is a further modification of the Sandison-Clark

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chamber (7, 8). Two features of the present chamber are of special significance for the studies described in this paper. One is the incorporation of a valve for the atraumatic introduction of test materials, an innovation originally proposed by Sanders (9). The other is the greater thickness of the tissue over the chamber table (70 to 100 μ.) which protects the vessels from minimal mechanical trauma, while retaining acceptable optical characteristics. These modifications prevent the non-specific inflammatory reactions which resulted from the introduction of materials into earlier chambers. Thus, for the first time, very early inflammatory phenomena of small magnitude can be related to the specific test substance and distinguished from non-specific effects of trauma. The present chamber has the further advantage that repeated injections of test materials can be made into it, so that the responses to different stimuli can be compared in the same blood vessels.

A modified experimental procedure was used in the present series of experiments in order to permit detailed observation of the early, transient reactions produced by some of the test materials. At the beginning of each experiment, approximately 0.004 ml of test material was introduced through the valve into the chamber by means of a syringe microburet (6). Observations were recorded at 2, 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes after injection. In many experiments, further observations were made after 120 minutes. Each observation was recorded in a manner similar to that described in the preceding paper (6). Two preselected segments of venules were examined, and the number of leucocytes "rolling" and "sticking" was recorded. A cell was considered to be rolling if it was seen moving slowly along the endothelium at a rate slower than that of the axial stream of the venule. A leucocyte was considered to be sticking if it was firmly adherent to the endothelial surface. It is generally supposed that rolling represents, to a lesser degree, the same phenomenon as sticking. Numbers of sticking or rolling cells greater than 100 were recorded as 100, because it was found that numbers of cells greater than this could not be counted accurately. The entire chamber was also examined for rolling and sticking of leucocytes and for evidence of leucocytic emigration. The amount of emigration was estimated and scored on an arbitrary scale, the details of which have been described previously (6). In the experiments with Hageman factor the inflammatory index (I.I.) was calculated (6) because it was found to provide a useful means of comparison of the effects of this substance with those of its controls.

Measurement of Increased Vascular Permeability.—Intradermal injections of test materials were made in the shaved backs of rabbits as previously described (6). Pontamine sky blue (E. I. DuPont De Neumours and Co., Inc., Wilmington, Delaware) was given intravenously as described before, except that the dye was injected immediately before the intradermal injections, rather than 30 minutes afterward as in the previous experiments. Intradermal injection sites were examined at 15, 30, 45, 60, 90, and 120 minutes after injection, and blue spots 5 mm or more in diameter were considered positive.

Saline.—Most substances were dissolved in pyrogen-free, sterile 0.15 M NaCl solution before use. The NaCl solution, which will hereafter be referred to as "saline" also was employed as a control material in both the ear chamber and the skin.

Hageman Factor.—Purified Hageman factor was prepared from citrated human plasma by a previously described method (10). The preparations used were purified 3000- to 5000-fold and contained 2 to 8 per cent protein; the non-protein solid was trisodium ethylenediamine tetra-acetic acid. The preparations were free of detectable amounts of other clotting factors, but were probably contaminated with traces of plasminogen. Originally, the preparations of Hageman factor were thought to be in the activated state. Subsequent studies demonstrated that only 1 to 8 per cent of the Hageman factor was in the activated form (2). In the experiments described herein, Hageman factor was dissolved in sterile barbital-saline buffer at pH 7.5 in a concentration of 4 mg per ml (3).

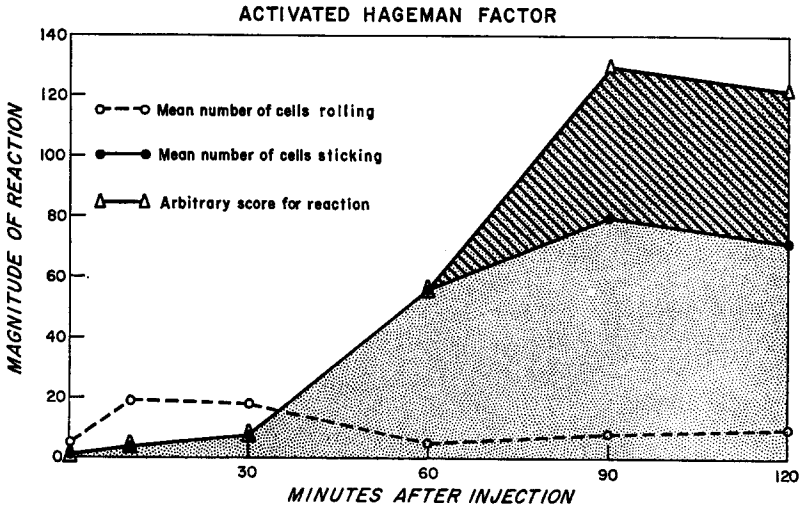
Ellagic Acid.—Ellagic acid (4,4′,5,5′,6,6′-hexahydroxydiphenic acid 2,6:2′,6′-dilactone), an activator of Hageman factor, was dissolved in barbital-saline buffer at a concentration of

2×10^{-4} M with the aid of a mechanical homogenizer. Both crude, commercial ellagic acid (K and K Laboratories Inc., Plainview, New York) and synthetic ellagic acid, prepared in the laboratories of the Department of Chemistry, Western Reserve University, were tested; the effects of each were indistinguishable. To prepare activated Hageman factor, equal parts of solutions of Hageman factor and ellagic acid were mixed, giving final concentrations of 2 mg per ml for Hageman factor, and 10^{-4} M for ellagic acid.

TABLE I

The Inflammatory Effects of Activated Hageman Factor

Ear chamber	Inflammatory index		
	Hageman factor	Ellagic acid	Hageman factor plus ellagic acid
382 R	191	140	616
382 R	14	10	266
746 L	0	–	505
700 L	–	5	86
805 L	5	0	440
762 L	95	5	230
806 L	0	0	750
746 R	0	–	–
612 L	0	–	–



TEXT-FIG. 1. Graphic representation of a typical reaction to activated Hageman factor. The stippled area beneath the solid line joined by triangles (score for the reaction) is the inflammatory index. The cross-hatched, stippled area between the total score and the number of cells sticking represents the portion of the inflammatory index contributed by the exudate. The number of cells rolling is not a factor in the numerical evaluation of the reaction. (See reference 6.)

Bradykinin.—Synthetic bradykinin ("BRS 640" Lot 013 12) was kindly donated by Sandoz Pharmaceuticals, Incorporated, Hanover, New Jersey.

RESULTS

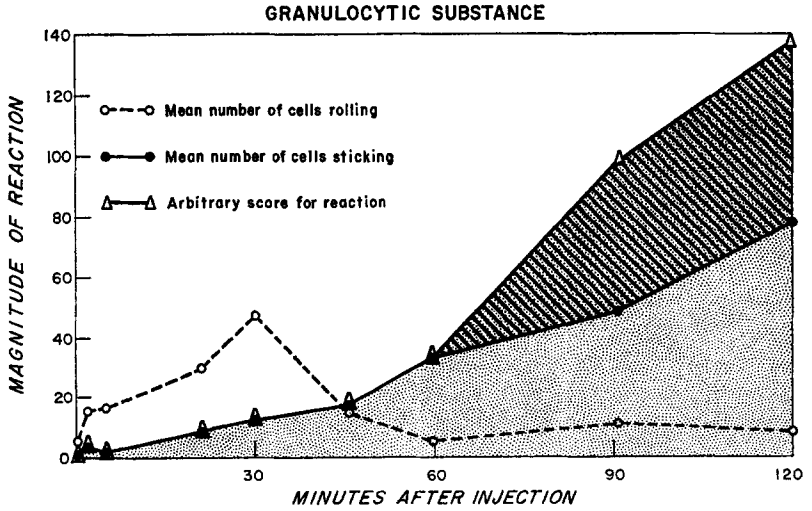
Saline.—Before use in experiments new ear chambers were "stabilized" by repeated injections of saline (6). After stabilization the maximum reaction to saline consisted of transient arteriolar dilatation and moderate rolling of

TABLE II

The Response to Hageman Factor plus Ellagic Acid in the Rabbit Ear Chamber

Ear chamber	Before injection				Minutes after injection																			
					10				30				60				90				120			
	R*	S‡	E§	A‖	R	S	E	A	R	S	E	A	R	S	E	A	R	S	E	A	R	S	E	A
382R		3	0	N		7	0	D		69	0	N		93	+	N						91	+	N
382R		1	0	D		6	0	D		5	0	D		25	0	D						100	+	D
746L	2	0	0	N	17	1	0	C	12	2	0	N	18	15	0	D	25	100	++	D	50	100	+++	D
700L	2	0	0	N	8	0	0	N	22	8	0	N	0	25	0	N					15	15	0	N
805L	6	1	0	N	11	2	0	D	10	2	0	D	7	2	0	D	10	6	0	D	8	30	0	D
762L	22	4	0	N	7	4	0	D	55	6	0	D	18	6	0	D	28	34	+	D	7	100	+	D
806L	3	0	0	C	2	2	0	N	6	46	0	D	5	90	+	D	8	100	++	D	35	70	+++	D

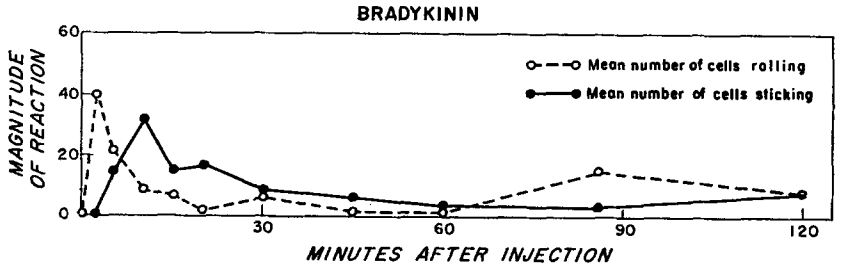
* Mean number of cells rolling in arbitrary venular segments.
‡ Mean number of cells sticking in arbitrary venular segments.
§ Amount of emigration (arbitrary scale of 0 to ++++).
‖ Arteriolar tone (N, normal, D, dilated, C, constricted).



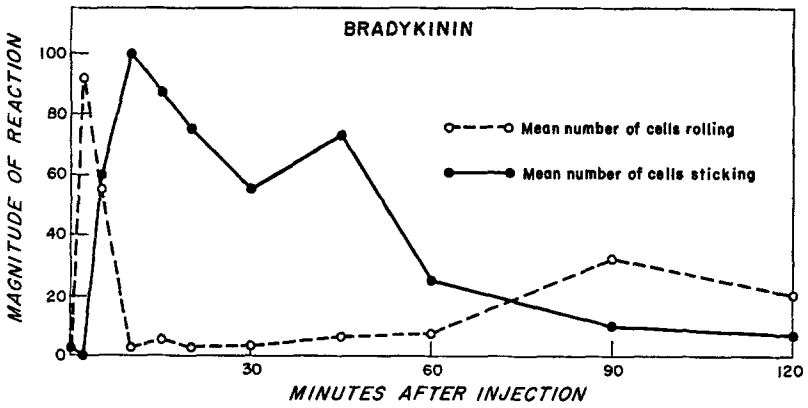
TEXT-FIG. 2. Graphic representation of a typical reaction to granulocytic substance. See explanation for Text-fig. 1.

leucocytes. In many stable chambers saline injection was followed by no detectable reaction. Significant sticking and emigration of leucocytes did not result from saline injection in stable chambers. In a few chambers non-specific reactions of significant proportions continued to occur; these chambers were not used for study of test materials.

Delayed reactions, such as those reported following injection of saline and



TEXT-FIG. 3. Graphic representation of a reaction to bradykinin (5 μg/ml) in the rabbit ear chamber.



TEXT-FIG. 4. Graphic representation of a reaction to bradykinin in the ear chamber. The same dose as in Text-fig. 3 was given to a different chamber illustrating the variability of response among chambers.

other substances by Hurley and Spector (11) were not observed in stable chambers during 3 hours of observation.

Inactive Hageman Factor.—In six of eight experiments inactive Hageman factor produced no more reaction than saline. Transient rolling of leucocytes occurred shortly after injection, but no significant fixed sticking occurred and no emigration of leucocytes was seen. In two experiments a somewhat greater reaction was observed (Table I) and there was minimal to moderate fixed sticking between 30 to 90 minutes after injection. No emigration of leucocytes occurred, however.

Ellagic Acid.—In five of six experiments 10^{-4} M ellagic acid produced no more reaction than did the control injection of saline (Fig. 1 *a*). In one experiment fixed sticking occurred between 30 and 90 minutes but this was minimal and no emigration of leucocytes occurred.

Hageman Factor Plus Ellagic Acid.—Purified Hageman factor, activated by 10^{-4}M ellagic acid, consistently evoked an inflammatory response which was delayed in onset and relatively prolonged (Text-fig. 1). Rolling of leucocytes often was increased at 10 minutes and usually reached its peak about 30 minutes after injection. By this time fixed sticking of leucocytes was often apparent. Sticking then gradually increased, usually becoming marked 45 to 60 minutes after injection (Fig. 1 *b*). The reaction generally reached its peak 90

TABLE III

Representative Experiments Illustrating the Effect of Bradykinin in the Rabbit Ear Chamber

Conc.	Ear chamber	Before injection				Minutes after injection																			
						10				30				60				90				120			
		R*	S‡	E§	A‖	R	S	E	A	R	S	E	A	R	S	E	A	R	S	E	A	R	S	E	A
μg per ml																									
5	746R	3	0	0	N	75	1	0	D	53	0	0	N	5	0	0	N	6	0	0	D	2	0	0	N
5	746L	2	0	0	N	50	5	0	D	18	11	0	N	5	6	0	N	7	13	0	N	7	10	0	D
5	612L	5	1	0	N	23	53	0	D	9	73	+	N	6	62	0	D	43	48	++	D	25	20	++	D
10	762L	10	1	0	N	85	16	0	D	35	41	0	N	8	0	0	N	14	1	0	N	54	6	0	N
10	805L	1	1	0	N	7	32	0	D	7	9	0	N	2	4	0	N	15	4	0	D	7	9	0	D

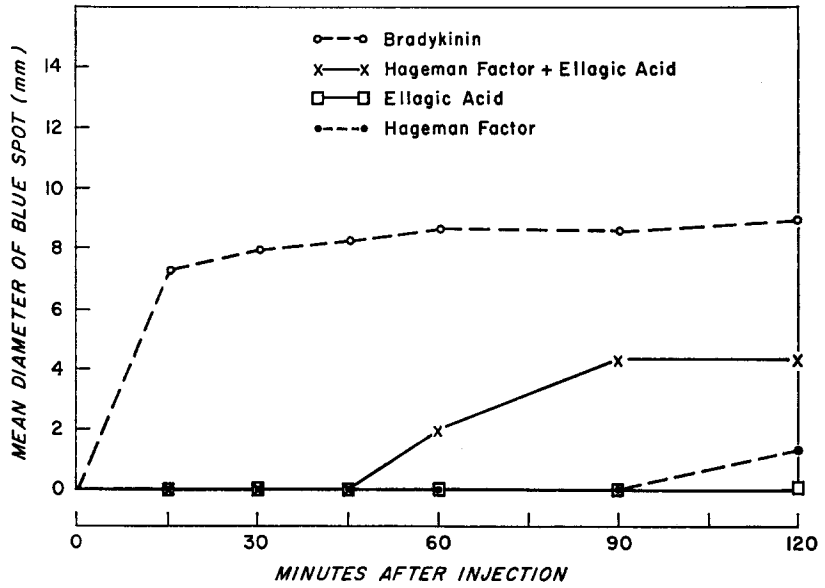
* Mean number of cells rolling in arbitrary venular segments.
‡ Mean number of cells sticking in arbitrary venular segments.
§ Amount of emigration (arbitrary scale of 0 to ++++).
‖ Arteriolar tone (N, normal; D, dilated; C, constricted).

to 120 minutes after injection. Significant emigration of leucocytes occurred in five of seven experiments (Table II). Arteriolar dilatation and rapid venous flow were also delayed in onset, occurring concomitantly with the leucocytic sticking and emigration. The inflammatory index in experiments with activated Hageman factor ranged from 86 to 750 with a mean of 413. This compared with a mean inflammatory index of 38 for inactive Hageman factor and of 27 for ellagic acid alone (Table I).

The pattern of response to activated Hageman factor (Text-fig. 1) was quite similar to that observed after the injection of "granulocytic substance" (Text-fig. 2) previously described from this laboratory. It should be noted that both are delayed reactions, usually reached their peaks about 90 minutes after injection.

Bradykinin.—The response of the microcirculation in the rabbit ear chamber to the injection of bradykinin (Text-figs. 3 and 4) was quite different from that

seen after the injection of activated Hageman factor (Text-fig. 1). Arteriolar dilatation appeared within a few seconds of the beginning of the injection but rarely persisted for more than 15 minutes. Soon after the onset of arteriolar dilatation, many leucocytes were seen rolling slowly along venular endothelium. This phenomenon usually was most prominent 2 to 5 minutes after injection. In the larger reactions many of the leucocytes began to adhere firmly to the endothelium within a few minutes of the onset of rolling. This sticking ordinarily reached its peak 10 to 20 minutes after injection, subsiding gradually thereafter (Fig. 2 *a* to 2 *d*). In the more intense reactions emigration of leucocytes was sometimes observed, usually apparent 20 to 30 minutes after injection (Fig. 3).



TEXT-FIG. 5. Comparison of permeability effects of bradykinin with Hageman factor and ellagic acid alone and Hageman factor activated by ellagic acid. Note the delay in onset of enhanced permeability with activated Hageman factor.

Different ear chambers varied markedly in reactivity (Table III), but each reacted consistently to a given dose of bradykinin. Several chambers exhibited significant leucocytic sticking after as little as 2 μg per ml, and detectable emigration after 4 to 5 μg per ml. On the other hand, an occasional chamber could be injected with as much as 40 or even 80 μg per ml with only moderate sticking and no significant emigration resulting. These differences were thought to depend largely on variable patterns of blood flow in individual chambers. In spite of the variability in magnitude, the pattern of reaction was quite characteristic. In all, thirty experiments were performed in ten different chambers,

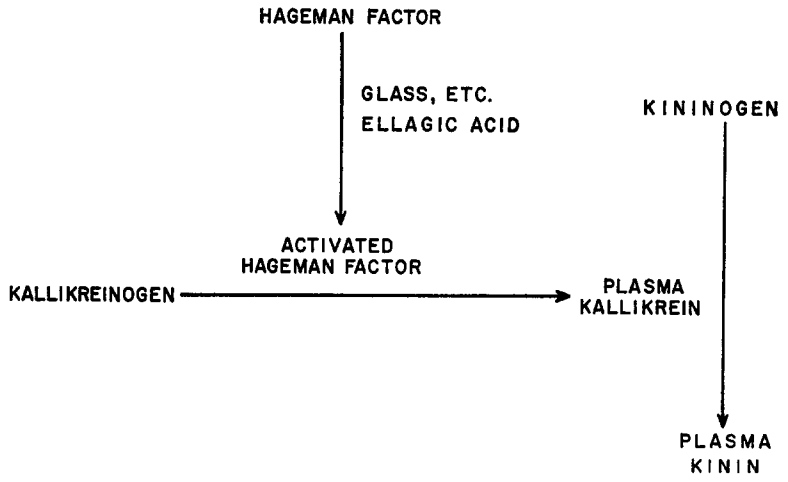
using concentrations of bradykinin ranging from 0.25 to 80 μg per ml. In twenty-four experiments in which the concentration of bradykinin was 4 μg per ml or greater, detectable emigration occurred in five. Emigration was not observed after concentrations of bradykinin lower than 4 μg per ml.

A Comparison of the Abilities of Bradykinin and Activated Hageman Factor to Increase Vascular Permeability in Rabbit Skin.—Bradykinin in concentrations as low as 0.0002 μg per ml increased vascular permeability in the skin of rabbits injected intravenously with pontamine sky blue. Bluing of the test area was evident within 5 to 10 minutes after intradermal injection of bradykinin, and intense bluing was noted after 30 minutes. In contrast a mixture of Hageman factor and ellagic acid caused a delayed increase in vascular permeability in rabbit skin (Text-fig. 5). No blue staining was usually evident until about 60 minutes after intradermal injection and became more intense after 90 to 120 minutes.

DISCUSSION

It has been demonstrated that activated Hageman factor causes significant and prolonged sticking of leucocytes to the endothelium of small blood vessels in rabbit ear chambers and that, subsequently, emigration of leucocytes can occur. Hageman factor in its inactive form (in terms of clot-promoting potency) has insignificant inflammatory effects.

Activated Hageman factor is thought to liberate a plasma kinin by initiating the series of reactions outlined in Text-fig. 6. The activated Hageman factor



TEXT-FIG. 6. Postulated role of activated Hageman factor in the liberation of plasma kinin by activation of the plasma kallikrein system.

apparently acts upon a plasma protein called kallikreinogen to liberate plasma kallikrein, a proteolytic enzyme. The kallikrein then catalyzes the release of a plasma kinin from kininogen, a circulating alpha-2 globulin. While the exact nature of this kinin has not been determined, it is probably bradykinin or a closely related polypeptide (1, 5).

The role of Hageman factor in naturally occurring inflammation is obscure. However, there is some evidence for the participation of kinins in the pathogenesis of certain inflammatory reactions. Roche e Silva (12) obtained a kinin-like substance from rat paws scalded at 45–48°C. Edery and Lewis (13) collected lymph from the hind limbs of dogs subjected to injury. They found increased kinin-forming activity, but no detectable preformed kinin. A recent study by Goldfinger and associates (14) provides evidence that kinins may be involved in the pathogenesis of several types of synovial inflammation. These workers recovered a kinin-like material from inflamed joints of patients with gout, rheumatoid arthritis, and psoriatic arthritis. When urate crystals were injected into the knees of a gouty volunteer, a 50-fold increase in kinin-like activity accompanied a striking inflammatory response. This latter finding is of particular interest since Kellermeyer and Breckenridge (15) have demonstrated that urate crystals can activate Hageman factor *in vitro*.

It is not known whether the inflammatory effects of activated Hageman factor are the result of kinin release, or whether they depend upon some other, presently unknown mechanism. If the delayed inflammatory reactions caused by activated Hageman factor are indeed finally mediated by the liberation of kinin, the present findings would seem to be of considerable theoretical interest. As the present studies indicate, the immediate and rather transient inflammatory response evoked by preformed bradykinin is quite dissimilar from the reaction elicited by activated Hageman factor, which is delayed, prolonged, and characterized by prominent sticking and emigration of leucocytes. It seems possible, then, that prolonged endogenous release of kinins may produce effects quite different from those observed after the usual single injection of exogenous kinin. If this is correct, kinins deserve further investigation as possible participants in the pathogenesis of the delayed phase of inflammation.

Another problem complicates the interpretation of the Hageman factor experiments. Although activated Hageman factor produces a delayed response both in the rabbit ear chamber and in rabbit skin, it results in early and transient bluing in the skin of the guinea pig (4, 16). It is not known whether Hageman factor evokes emigration of leucocytes in this animal. This discrepancy recalls the species difference noted by Wilhelm and associates (17) in studying globulin permeability factors. In the rabbit, homologous permeability factor evoked a delayed and prolonged increase in permeability. In contrast, homologous permeability factors produced immediate, transient responses in the rat and guinea pig. Curiously, rabbit permeability factor evoked immediate

transient responses in rats and guinea pigs, and permeability factors from the latter two species resulted in immediate, transient permeability increases in the rabbit. The reasons for these species differences are totally unknown, and the two examples are perhaps not comparable since the Hageman factor used in the current experiments was not homologous. Nevertheless, generalization between species is clearly hazardous on the basis of the present information.

The similarities of the reactions evoked by activated Hageman factor and granulocytic substance (6) raised the question of the possible identity of the two substances. In preliminary experiments, granulocytic substance was tested for clot-promoting activity and, specifically, for ability to activate PTA. Clot-promoting activity could not be demonstrated because of the presence of an anticoagulant material in granulocytic substance. The mechanism of this anticoagulant activity is currently being studied. Although Hageman factor and granulocytic substance are clearly not the same, it remains possible that they act in a similar fashion by liberating a common mediator.

SUMMARY

Activated Hageman factor, when injected into the rabbit ear chamber, produces a delayed and prolonged inflammatory response characterized by prominent sticking and emigration of leucocytes. In contrast, preformed bradykinin evokes an immediate and more transient response in which leucocytic emigration occurs less frequently. It is concluded that either Hageman factor produces its inflammatory effects by mechanisms other than kinin release, or bradykinin released endogenously has effects quite different from those resulting from a single injection of the exogenous material.

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EXPLANATION OF PLATES

PLATE 60

FIG. 1 *a*. Forty-five minutes after injection of ellagic acid alone. Only a few rolling leucocytes are evident. × 700.

FIG. 1 *b*. Forty-five minutes after injection of a mixture of Hageman factor and ellagic acid. There is extensive sticking of leucocytes to venular endothelium. × 700.

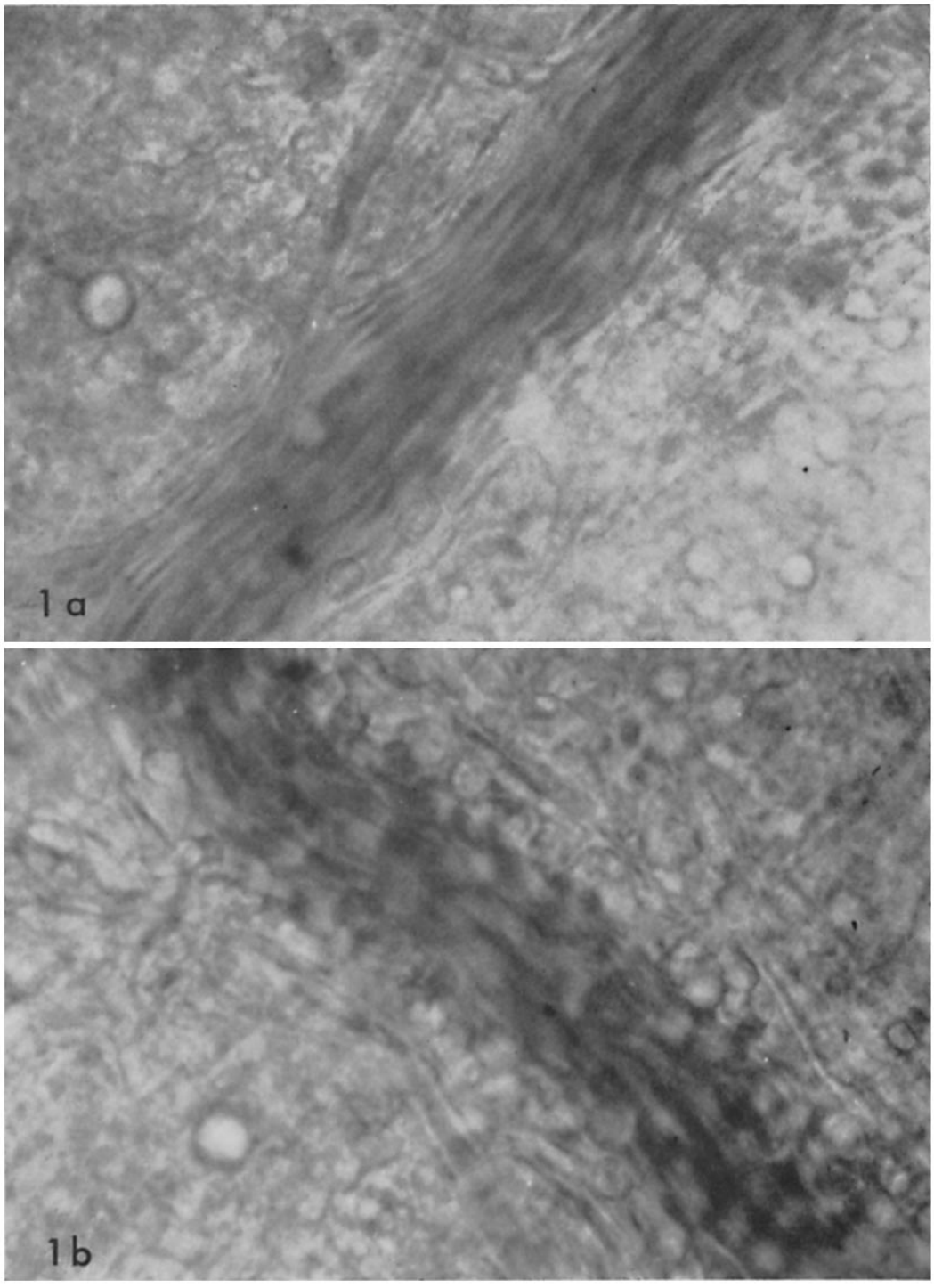

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PLATE 61

FIGS. 2 *a* to2 *d*. Sequential changes of arteriole (*A*) and venules of rabbit car chamber before and after injection of bradykinin 5 μg per ml. $\times$ 200.

FIG. 2 *a*. Before injection. Appearance of vessels is normal

FIG. 2 *b*. Three minutes after bradykinin. Arteriole (*A*) is widely dilated, venular flow is rapid, and many rolling leucocytes are apparent.

FIG. 2 *c*. Twelve minutes after bradykinin. Marked arteriolar dilatation persists. Leucocytes can be seen sticking to venular endothelium.

FIG. 2 *d*. Seventeen minutes after bradykinin. Arteriole (*A*) is only slightly dilated. Only a few leucocytes are still sticking.

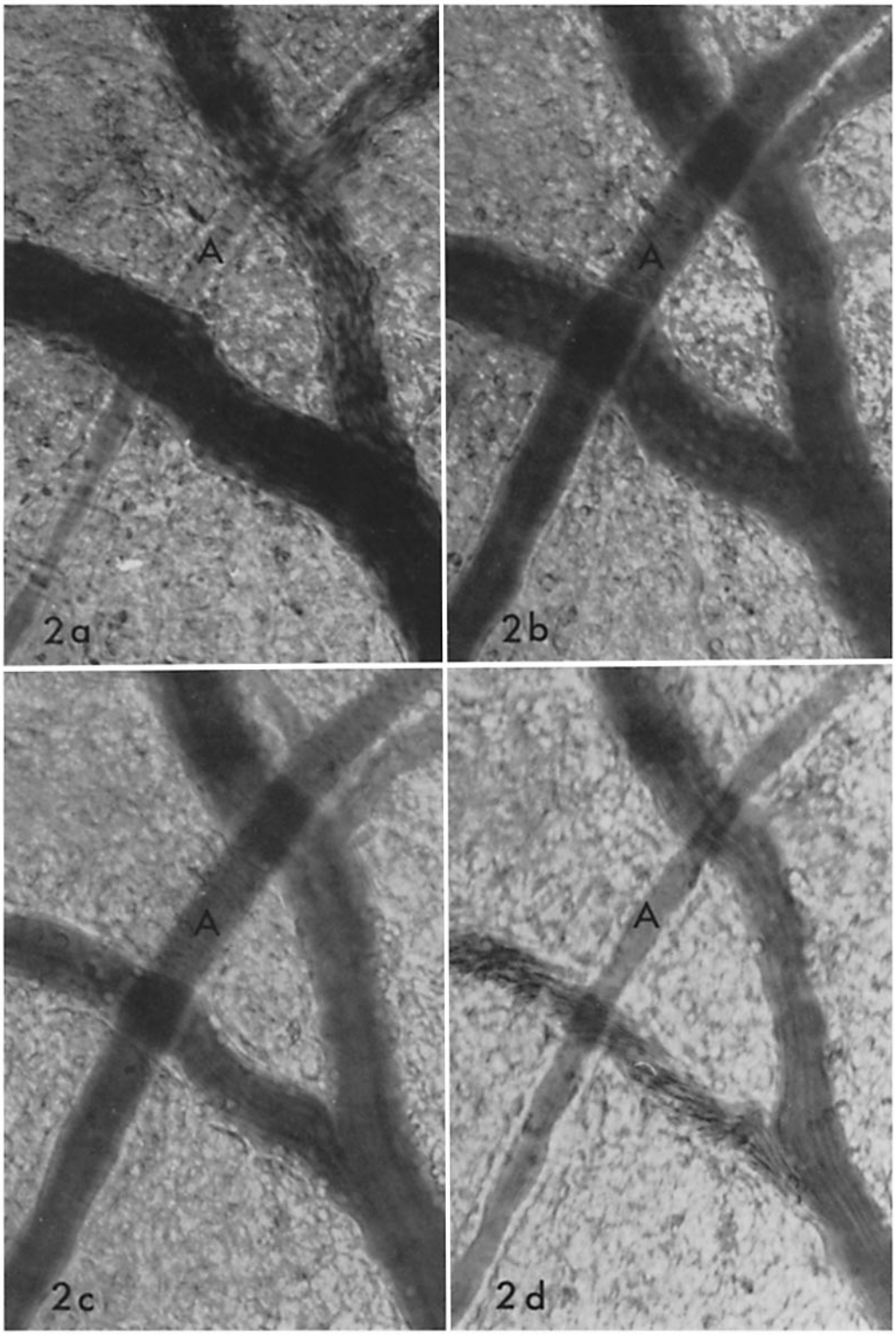

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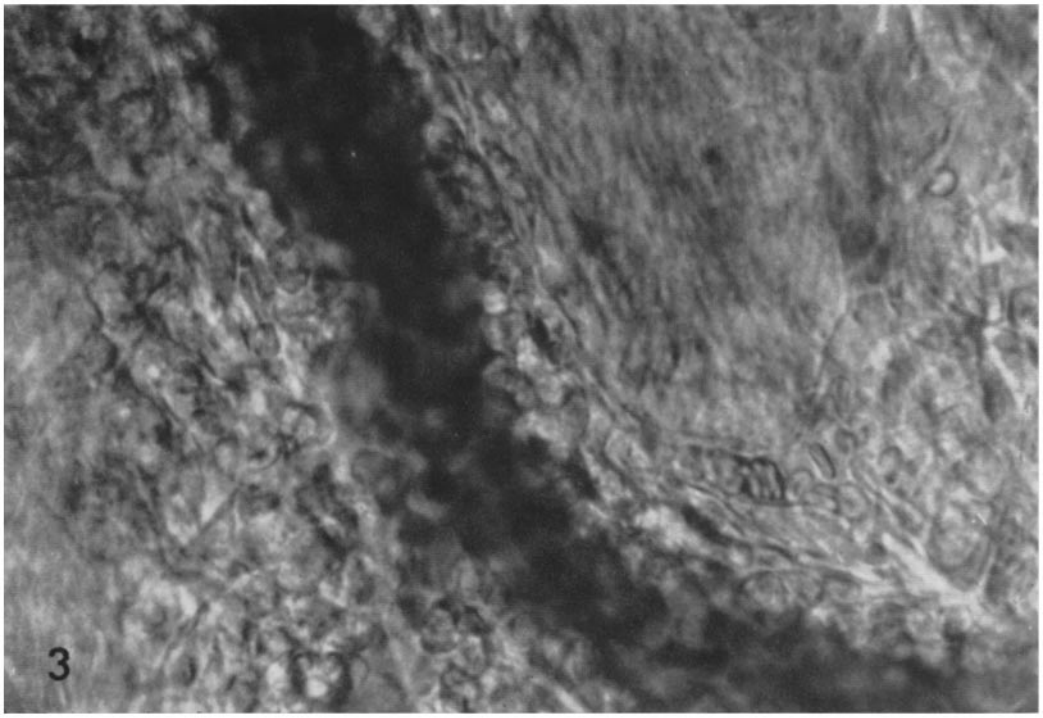
PLATE 62

FIG. 3 Extensive sticking of leucocytes to venular endothelium 20 minutes after injection of bradykinin (5 μg per ml.). Even when the response to a single injection of bradykinin was this marked, it was quite transient, largely subsiding within an hour. $\times$ 680.



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