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EFFECT OF HEXADIMETHRINE BROMIDE ON PLASMA KININ FORMATION, HYDROLYSIS OF *p*-TOSYL-L-ARGININE METHYL ESTER AND FIBRINOLYSIS

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The antiheparin agent hexadimethrine bromide, in concentrations of 20 to 200 μg/ml., inhibited the activation by active Hageman factor of the plasma enzyme which releases kinin from substrate. Once activated, this kinin-forming enzyme was not consistently inhibited by hexadimethrine in a concentration of 1 mg/ml. Surfaces which induce kinin formation by activating Hageman factor in plasma (glass, kaolin, celite, barium carbonate and carboxymethylcellulose) were inactivated by bathing in aqueous solutions of hexadimethrine. The effects of hexadimethrine on Hageman factor and on glass were not abolished by amounts of heparin which neutralize most other actions of hexadimethrine. Hexadimethrine prevented the activation by kaolin, but not by streptokinase, of *p*-tosyl-L-arginine methyl ester-splitting and fibrinolytic factors in plasma; once these enzymes were activated by kaolin, they could not be inhibited by hexadimethrine. Hexadimethrine, given locally or intravenously into guinea-pigs, reduced the increase in capillary permeability produced by intracutaneous injections of kaolin suspensions.

Intrinsic kinin formation in human plasma appears to be usually initiated by the activation of Hageman factor, an enzyme which also initiates blood clotting and fibrinolysis (Ratnoff & Colopy, 1955; Soulier & Prou-Wartelle, 1959; Margolis, 1960; Eisen & Keele, 1960). Hageman factor leads to kinin formation by activating another enzyme which then acts directly on a kinin-yielding substrate (Fig. 1). The activation of the fibrinolytic system by Hageman factor is probably responsible for only a small part of the kinin formation in plasma, since even much greater fibrinolytic activity induced in plasma by streptokinase produces only little kinin (Eisen, 1961, 1963b).

Armstrong & Stewart (1962) have reported that hexadimethrine bromide, which is used clinically for neutralizing the anticoagulant effects of heparin (Preston, 1952), inhibits certain modes of plasma kinin formation. The present study suggests that hexadimethrine achieves this effect by interfering with the activation and action of Hageman factor. This effect of hexadimethrine has also been demonstrated on esterolytic and fibrinolytic activity initiated by Hageman factor. Moreover, hexadimethrine suppresses *in vivo* the increase in capillary permeability produced by kaolin.

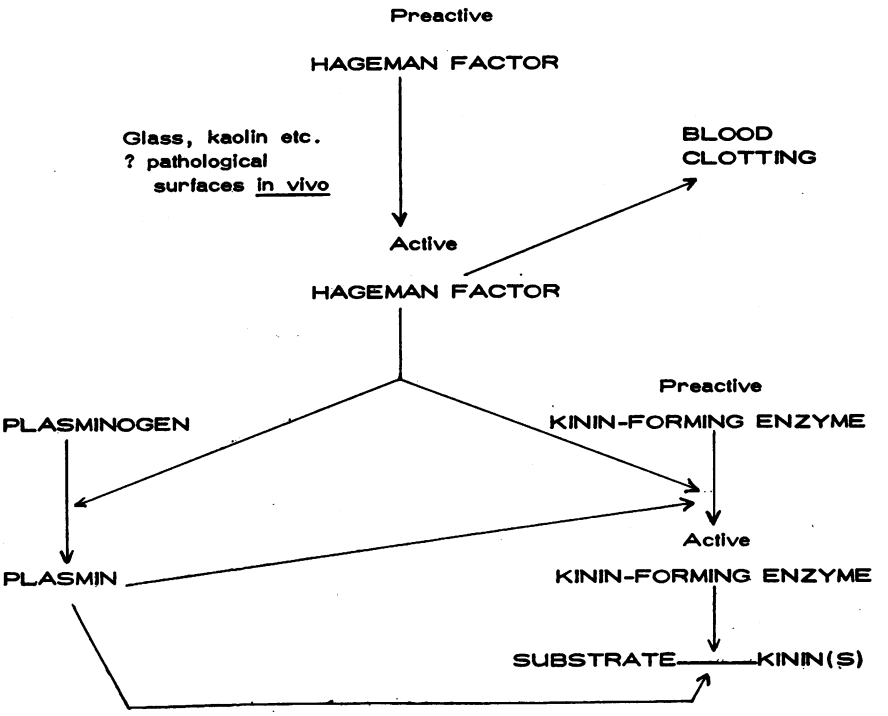

Fig. 1. Diagram of possible relationship of intrinsic plasma kinin formation and fibrinolysis.

METHODS

Materials and drugs. These were: ballotini, glass beads of 0.1 mm diameter (English Glass Co., Leicester), approximate surface of 200 cm^2/g; kaolin light (B.D.H.), approximate surface of 7.5 m^2/g (Margolis, 1963); Michaelis' veronal acetate: saline buffer with *p*H 7.4 (Biggs & Macfarlane, 1957); tris-(hydroxymethyl)aminomethane (B.D.H.) buffer solutions prepared from a 1.0 M stock solution and hydrochloric acid; hexadimethrine bromide (Polybrene, Abbott), from ampoules containing 10 ml. of a 10 mg/ml. solution, with average molecular weight of 6,000; benzethonium chloride (Phemerol Chloride, Parke-Davis); and Cetrimide (B.P. Add.), a mixture of dodecyl-, tetradecyl- and hexadecyltrimethylammonium bromides; synthetic bradykinin; freeze-dried plasma from a patient with Hageman trait (congenital deficiency of Hageman factor); preparations of purified plasma enzymes: (A) acting mainly directly on the kinin-yielding substrate in plasma and therefore forming kinin at similar rates in fresh intact plasma and in heated plasma: human plasma kallikrein, 0.35 Frey units/mg; (B) containing an activator of the kinin-forming enzyme in plasma, and forming kinin much faster in intact plasma than in heated plasma: human "permeability factor (Fraction B1R) containing kininogenase activity" and guinea-pig "permeability factor (Fraction G2/1R) without kininogenase activity" (Mason & Miles, 1962); human "plasma kinin-forming substance"; Thrombolysin (human plasmin with plasminogen activator, Merck, Sharp & Dohme); and purified activated human Hageman factor.

Plasma and serum. These were prepared from fresh blood using polyethylene or siliconed glass equipment. Plasma or serum which had not been in contact with glass is referred to as "intact plasma." "Substrate-depleted plasma" ("B-depleted plasma," Margolis, 1958) was prepared in the following way: intact plasma was shaken with ballotini, 0.17 g/ml. for 10 min, then separated from the beads, put into polyethylene vessels and left at room temperature for 6 to 8 hr. In this way a part of the kinin-yielding substrate in plasma was

consumed, so that renewed contact with glass produced only kinin-forming activity and no detectable kinin.

Heated plasma. Plasma was adsorbed with barium sulphate (100 mg/ml.) for 10 min, separated, and then heated. After heating denatured protein was removed by centrifugation, and the plasma dialysed against 0.9% saline at 4° C. After incubation at 56° C for 3 hr, human plasma contains Hageman factor and kinin-yielding substrate, but has lost the bulk of kinin-forming enzyme ; after incubation at 61° C for 20 min, only substrate and no Hageman factor or kinin-forming enzyme is found (Eisen, 1963a).

Adsorption of plasma factors on to glass. Ballotini were immersed for 3 to 10 min in intact plasma to adsorb Hageman factor and kinin-forming enzyme, or in heated plasma (56° C for 3 hr) to adsorb Hageman factor only (Eisen, 1963a). They were then thoroughly washed with 0.9% saline which did not remove these adsorbed enzymes. Adsorbed Hageman factor could be demonstrated by its activating effect on Hageman trait plasma, and adsorbed kinin-forming enzyme by its capacity to activate heated plasma.

Plasma was treated with acid by the method of Alkjaersig, Fletcher & Sherry (1959), and with cold acetone by Lassen's (1958) method. The euglobulin fraction was separated according to the method of Milstone (1941).

Kinin formation. This was assessed on single horns of isolated uteri from virgin rats previously treated with stilboestrol (50 μg/100 g). Auxotonic contractions (Paton, 1957 ; Eisen, 1963a) were measured. Experiments were performed in an automatic biological assay apparatus (Casella Electronics) which delivered three or four standard concentrations of synthetic bradykinin. Test solutions were applied manually. All agents were applied for 1 min periods at 5 min intervals, in a 5 ml. organ-bath.

Hydrolysis of p-*tosyl*-L-*arginine methyl ester (Hoffman-La Roche).* This was measured as described by Roberts (1958).

Fibrinolysis. This was measured by the dissolution of standard clots made of plasma, or of the euglobulin fraction with bovine fibrinogen (Armour Laboratories). This fibrinogen was also used to prepare unheated and heated (85° C for 35 min) fibrin plates (Astrup & Müllertz, 1952). Thrombin (Maw) was used for clotting.

The effect of kaolin on fibrinolysis was measured using the " plasminoplastin generation test " (Iatridis & Ferguson, 1961, 1962) with minor modifications.

Effects on capillary permeability. These were studied following the method developed by Miles & Wilhelm (1955). Animals were anaesthetized with pentobarbitone sodium. Pontamine Sky Blue, 0.2 ml./100 g body weight of a 2.5% solution, and other intravenous injections were given through cannulae tied into an external jugular vein of guinea-pigs, and in rats into a tail vein. Test solutions (0.1 ml.) were injected intracutaneously in random order (Latin Square design).

RESULTS

Effects of hexadimethrine on kinin formation

Inhibition by hexadimethrine (10^{-8} to 10^{-3} g/ml.) of kinin formation induced in plasma by dilution, sodium edetate, ε-amino caproic acid, contact with glass and separation of the pseudoglobulin fraction was first reported by Armstrong & Stewart (1962). In the work reported here hexadimethrine (10^{-5} to 10^{-3} g/ml.) also inhibited the kinin formation which occurs in plasma after exposure to *p*H 2 for 15 min, or after precipitation of plasma with cold acetone and subsequent solution in water. The kinin formation in plasma treated with acid and diluted 10 times was reduced by hexadimethrine (1 mg/ml.) to 10 to 20% of the control rate, and by 0.05 to 0.1 mg/ml. by about half. Similar degrees of inhibition were obtained in acetone-treated plasma.

Effect of hexadimethrine on kinin-forming enzyme. The inhibitory effect of hexadimethrine was analysed on the basis of the scheme of plasma kinin formation shown in Fig. 1. It was shown that hexadimethrine mainly inhibited the *activation* of the kinin-forming enzyme, and not the *action* of this enzyme on substrate. Activation and activity were separated as described by Margolis (1958). When plasma depleted of substrate was shaken with ballotini for 2 min, no kinin was found on separation from the glass (Fig. 2,B), although kinin-forming activity had been generated and could be shown by incubating such plasma with a plasma rich in

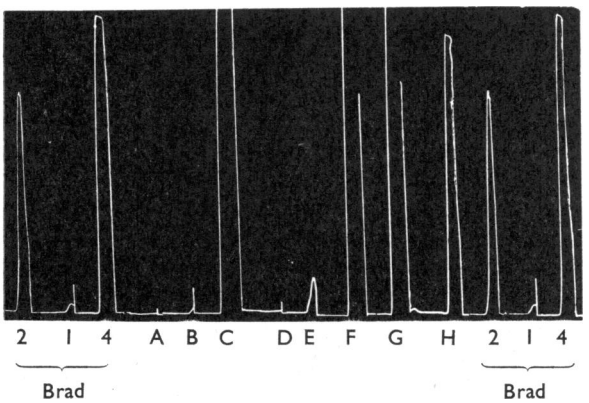

Fig. 2. Inhibition by hexadimethrine of mainly the activation of kinin-forming factors in plasma and not their activity. Rat uterus. Brad, responses to bradykinin 1, 2 and 4 ng/ml.; A, 0.2 ml. of substrate- (kininogen)- depleted plasma (diluted four times); B, as A, but shaken with ballotini (1 g/ml.) for 3 min, then separated and 0.1 ml. tested; C, as B, but mixed with an equal volume of heated plasma (at 56° C for 3 hr), and 0.15 ml. of the mixture tested after 3 min of incubation; D and E, as C, but the ballotini activation of substrate-depleted plasma was in the presence of hexadimethrine (10 μg/ml. D, or 5 μg/ml., E); F, G and H, as C, but the heated plasma contained hexadimethrine (10 μg/ml., F, 100 μg/ml., G, or 1,000 μg/ml., H).

substrate, for example intact or heated plasma (Fig. 2,C). When present during the activation by ballotini of plasma depleted of substrate, hexadimethrine (5 to 10 μg/ml.) significantly inhibited the generation of kinin-forming activity (Fig. 2,D,E). In contrast, the effect of already generated kinin-forming activity was not inhibited when hexadimethrine was added in this concentration range with the substrate-rich plasma (Fig. 2, F,G), and even a concentration of 1 mg/ml. reduced little or none of the kinin formed (Fig. 2,H).

Formation of kinin by purified human plasma kallikrein (Webster & Pierce, 1960), a substance which acted directly on substrate, was not inhibited by hexadimethrine (up to 1 mg/ml.). This result confirmed that already active kinin-forming enzyme is not attacked by hexadimethrine.

Effect of hexadimethrine on Hageman factor. It is clear from Fig. 1 that hexadimethrine could prevent the activation of kinin-forming enzyme in plasma by interfering either with the activation of Hageman factor or with the action of active Hageman factor on the precursor of the kinin-forming enzyme. Since the experiment

illustrated in Fig. 2 did not distinguish between these two stages, an attempt was made to study them separately. In these experiments, the effect of Hageman factor was assessed by the formation of kinin it induced in Hageman trait plasma.

Solutions of purified active Hageman factor (Ratnoff & Davie, 1962) were incubated with Hageman trait plasma so that the resulting formation of kinin was of similar magnitude to that in the tests with enzymes which act directly on substrate. The purified factor was inhibited by hexadimethrine (20 to 200 μg/ml., Fig. 3,*a*). The same hexadimethrine concentrations inhibited crude Hageman factor adsorbed onto ballotini from heated plasma (Fig. 3,*b*).

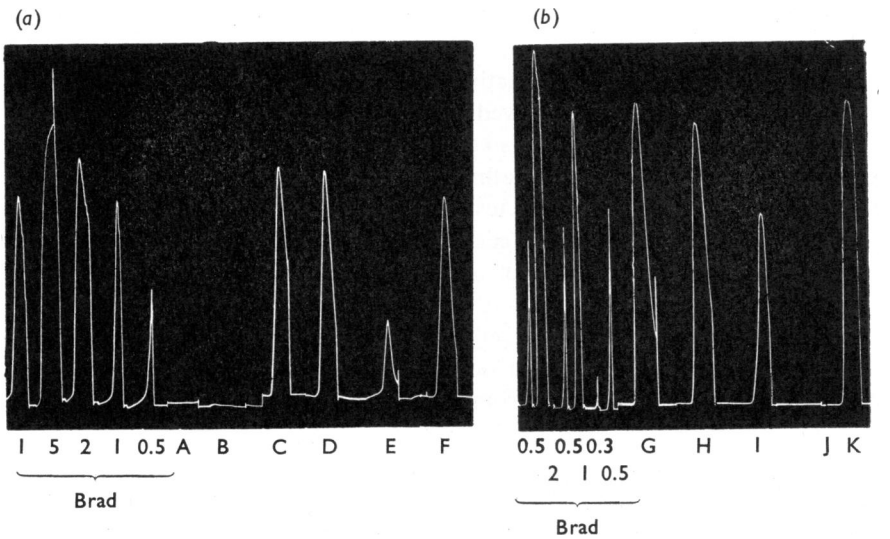

Fig. 3. Inhibition by hexadimethrine of responses of rat uterus to active Hageman factor.

(*a*), tests with purified Hageman factor; Brad, responses to bradykinin (0.5, 1, 2 and 5 ng/ml.; A, Hageman trait plasma; B, Hageman factor in 1% solution of bovine albumin; C to F, Hageman trait plasma (A) was tested after 5 min of incubation with an equal volume of Hageman factor (B). Before mixing with the plasma, Hageman factor was incubated for 20 min with the following concentrations of hexadimethrine in μg/ml.: C, 0; D, 2; E, 100; and F, 20. Tests A to F used a volume of 0.1 ml.

(*b*), tests with crude adsorbed Hageman factor. Brad, responses to bradykinin (0.3, 0.5, 1 and 2 ng/ml., drum running at half-speed). Ballotini were immersed in heated plasma (at 56° C for 3 hr) from which they adsorbed Hageman factor. After washing, they were shaken with Hageman trait plasma which was separated after 3 min and 0.1 ml. was tested. This Hageman trait plasma had previously been incubated with the following concentrations of hexadimethrine (in μg/ml.): G and K, 0; H, 10; I, 50; and J, 100.

Human and guinea-pig permeability factor, kinin-forming substance from human plasma, and commercial human plasmin with plasminogen activator (Thrombolysin) produced plasma kinin in a similar way as did Hageman factor, that is, by activating kinin-forming enzyme in plasma and not by a direct action on substrate. However, these substances were not clearly inhibited by hexadimethrine in concentrations up to 1 mg/ml., and were thus less sensitive than active Hageman factor.

The activation of Hageman factor. This process appeared to be inhibited by the same concentrations of hexadimethrine (20 to 200 μg/ml.) as was its activity. However, it became obvious in the course of these experiments that glass immersed in plasmas which contained hexadimethrine not only failed to adsorb Hageman factor, but also lost the capacity of clean glass to activate normal plasma. Moreover, glass which was coated with Hageman factor first, and then washed with hexadimethrine solutions, also lost its activating properties. Hexadimethrine had the same effect on glass which had adsorbed Hageman factor with kinin-forming enzyme from intact plasma.

Effects of hexadimethrine on glass. These effects appeared to be due mainly to a direct action of hexadimethrine on glass, and not to the formation of an inactive complex of adsorbed enzymes and inhibitor, since aqueous solutions of hexadimethrine also abolished the activating power of clean glass. Consistent "inactivation" of glass was achieved by hexadimethrine (0.1 to 1.0 mg/ml., but lower concentrations were usually also effective). Indeed, glass was rendered inactive by previous treatment with hexadimethrine in concentrations which had no detectable inhibitory effect when present in the test plasma. With hexadimethrine in concentrations of 100 μg/ml. or higher, the effect on glass was rapid, and with lower concentrations more gradual. Inactivation of a glass surface was still evident a week after the application of hexadimethrine. It was not abolished by thorough washing with tap or distilled water, with acetone, ether or ethanol, or by firm wiping with gauze, but the glass could be restored to normal by washing with N-hydrochloric acid, N-sodium hydroxide solution or chromic acid (Fig. 4). Heating at 115° C for 1 hr

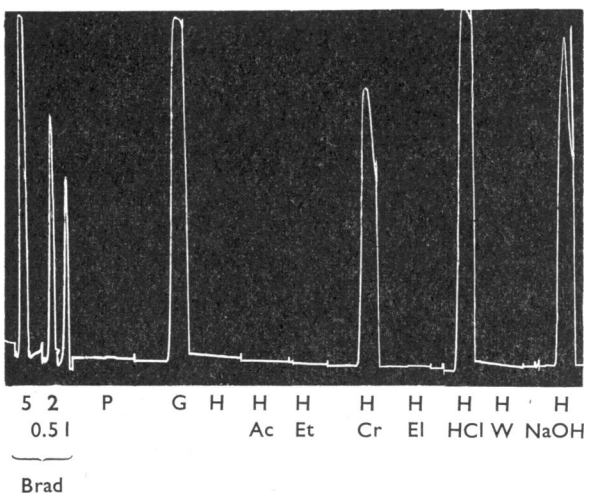

Fig. 4. Effect of chemical and mechanical cleaning on glass treated with hexadimethrine. Brad, responses to bradykinin (0.5, 1, 2 and 5 ng/ml., drum running at half-speed); P, 0.1 ml. of intact plasma. In the remaining tests, 0.1 ml. of plasma was tested after 4 min of shaking in glass vessels; these were clean (G) or previously treated with hexadimethrine (H) and then washed with acetone (Ac), ether (Et), chromic acid (Cr), ethanol (El), N-hydrochloric acid (HCl), N-sodium hydroxide solution (NaOH), or wiped with gauze (W).

also abolished the effect of hexadimethrine, whilst routine washing with the alkaline Haemo-Sol (Meinecke) inhibited it only partially.

Glass surfaces treated with hexadimethrine remained inactive even when brought into contact with several consecutive samples of plasma. Exposure of a plasma sample to glass treated with hexadimethrine did not reduce the subsequent response of the same sample to clean glass. Thus, there was no evidence that adsorbed hexadimethrine was displaced and taken up by plasma.

Hexadimethrine reduced the activating power not only of glass, kaolin and celite, but also of some non-silicate materials, such as carboxymethylcellulose and barium carbonate, which induce plasma kinin formation.

The described results suggested that hexadimethrine prevented plasma kinin formation induced by contact mainly by acting on the activating surface, and not on the enzymes in plasma. It seemed therefore of interest to examine how such treatment of glass affected those modes of intrinsic plasma kinin formation which also proceed in vessels with relatively inert surfaces, for example, polyethylene or siliconed glass. The formation of kinin which occurs in human plasma after dilution, exposure to *p*H 2 for 15 min, precipitation with acetone and subsequent solution, or

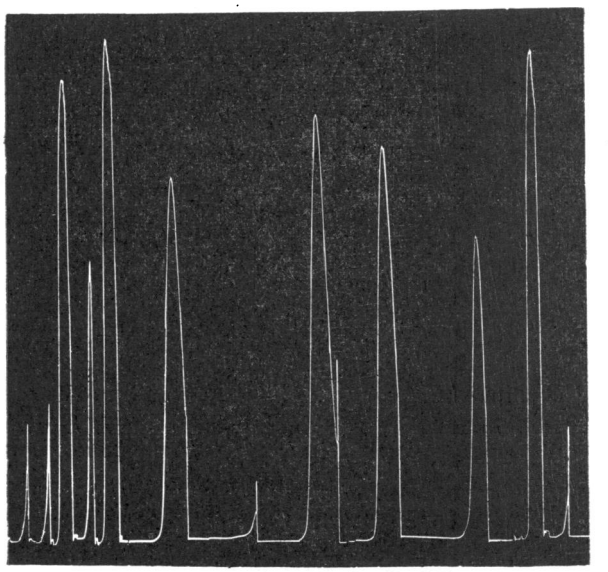

Fig. 5. Reduction by previous exposure of vessels to hexadimethrine of kinin formation in plasma treated with acid. Rat uterus. Brad, responses to bradykinin (0.5, 1 and 2 ng/ml., drum running at half-speed). Plasma was acidified to *p*H 2 for 15 min, then neutralized and diluted to twenty times its original volume at zero time. Aliquots were put into glass vessels previously siliconed (GS), coated with hexadimethrine (1 mg/ml., GH), or left clean (G). Times and volumes for tests as indicated.

incubation with ε-aminocaproic acid, proceeded in glass treated with hexadimethrine at a slower rate than in siliconed glass or in polyethylene (Fig. 5). Again, there was no evidence that hexadimethrine adsorbed on glass was taken up by the plasma preparations. For example, samples of diluted plasma from siliconed vessels and from those treated with hexadimethrine formed kinin at similar fast rates when transferred into clean glass vessels.

Contact with clean glass did not enhance the kinin-forming potency of thrombolysin, of purified plasma kallikrein, of kinin-forming substance or of permeability factor, nor did glass acquire an activating agent from these substances. Accordingly, their effect was the same in the presence of clean glass and of glass treated with hexadimethrine. Purified Hageman factor, on the other hand, was adsorbed by clean glass, and 10 min contact with ballotini (1 g/ml.) approximately doubled the potency of the preparation. This increase in potency was not produced by ballotini previously treated with hexadimethrine.

Effects of heparin on the inhibitory action of hexadimethrine. Studies on blood clotting and on several *in vivo* actions have shown that 0.7 to 1.0 mg of hexadimethrine neutralizes 1.0 mg of heparin and *vice versa.* (Kimura, Young, Stein & Richards, 1959). The effects of hexadimethrine on kinin formation were not abolished by heparin in this proportion, although three- to five-fold excess of heparin counteracted incompletely the effect of hexadimethrine on plasma activation by glass, acid or dilution, and on purified Hageman factor. The same results were obtained whether heparin was added to the kinin-forming system before, after, or at the same time as hexadimethrine. The effect of hexadimethrine on glass was also very resistant to heparin. Hexadimethrine reduced the activating power of glass surfaces even when mixed with a tenfold excess of heparin. Washing with heparin did not restore the normal activating properties of glass treated with hexadimethrine.

It was noticed in these experiments that a fine white precipitate rapidly formed when aqueous solutions of hexadimethrine and heparin were mixed. The precipitate was persisent with an excess or a neutralizing amount of heparin, but dissolved slowly in an excess of hexadimethrine.

Comparison of hexadimethrine with other compounds. Since the effects of hexadimethrine on activation and on activity of the kinin-forming enzyme were clearly different, an attempt was made to see whether soya bean trypsin inhibitor acted in a similar way. In contrast to hexadimethrine, soya bean trypsin inhibitor inhibited mainly the activity and not the activation of the kinin-forming enzyme (Fig. 6). The activating power of glass was not reduced by bathing in aqueous solutions of soya bean trypsin inhibitor.

Armstrong & Stewart (1962) associated the inhibitory effect of hexadimethrine with the positive charges on its quaternary ammonium groups. The effects on glass of other compounds carrying positive charges were therefore examined. The anti-heparin agents protamine sulphate and toluidine blue inactivated glass, but were less efficent than hexadimethrine. The inactivation of glass by penta-, hexa- and decamethonium iodide, and by the cationic detergents cetrimide and benzethonium chloride, was very slight and not consistent.

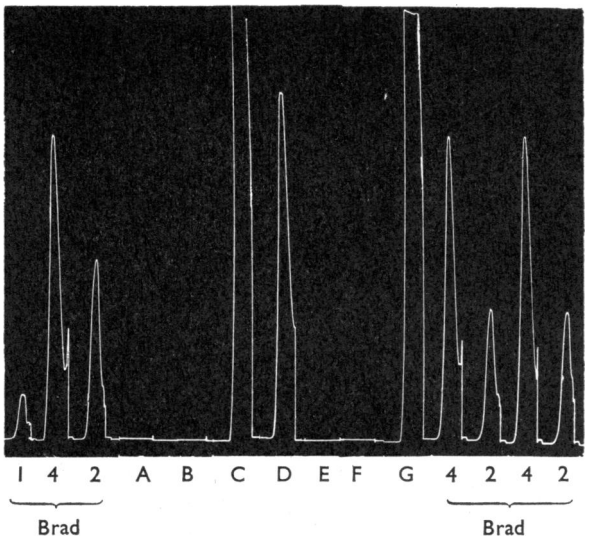

Fig. 6. Inhibition by soya bean trypsin inhibitor mainly of activity and not of activation of the kinin-forming plasma factors responsive to glass. Rat uterus. Brad, responses to bradykinin (1, 2 and 4 ng/ml.). In tests A to G, 0.1 ml. volumes were used. A, heated plasma (at 56° C for 3 hr). In tests B to G, plasma was shaken for 4 min with ballotini (1 g/ml.) and then separated and tested. B, heated plasma, clean ballotini; C, intact plasma, clean ballotini; D, heated plasma, ballotini from C; E, heated plasma with soya bean trypsin inhibitor (0.5 mg/ml.), ballotini from C; F, intact plasma with soya bean trypsin inhibitor (0.5 mg/ml.), clean ballotini; G, heated plasma, ballotini from F. Note that ballotini adsorbed from intact plasma (C) active Hageman factor and kinin-forming enzyme which were demonstrated on heated plasma (D). The presence of soya bean trypsin inhibitor in intact plasma inhibited the release of kinin (F), but did not prevent the adsorption/activation of these enzymes (G).

Effects on p-*tosyl-*L*-arginine methyl ester hydrolysis*

Exposure to silica surfaces such as kaolin induces in human plasma or serum activity for splitting *p*-tosyl-L-arginine methyl ester (Eisen, 1963b). Studies on the influence of hexadimethrine on this effect suggested that the activation, but not the activity of the responsible enzyme, was inhibited. Hydrolysis of *p*-tosyl-L-arginine methyl ester was suppressed only when hexadimethrine was added *before* kaolin to plasma. Fig. 7 shows that concentrations of hexadimethrine which effectively inhibited the activation by kaolin of enzymes which split *p*-tosyl-L-arginine methyl ester had no effect on their activation by streptokinase. Added *after* kaolin, even the highest hexadimethrine concentrations reduced hydrolysis of *p*-tosyl-L-arginine methyl ester little or not at all (Fig. 8).

Kaolin previously immersed in hexadimethrine and then washed had also less effect on plasma than had clean kaolin. However, the hexadimethrine coating was more easily removed by washing from kaolin than from glass.

Previous incubation of plasma with *p*-tosyl-L-arginine methyl ester did not oppose the inhibition of kaolin activation by hexadimethrine (Table 1). There was thus no evidence that *p*-tosyl-L-arginine methyl ester acted as a protective substrate which

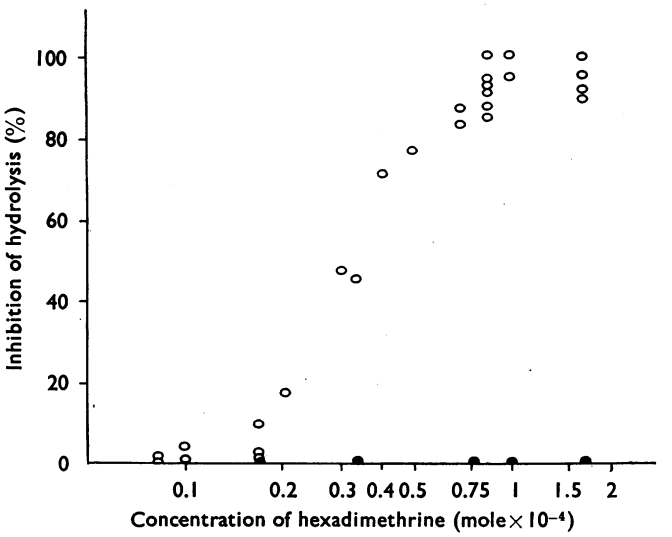

Fig. 7. Prevention by hexadimethrine of activation of *p*-tosyl-L-arginine methyl ester-splitting plasma enzymes by kaolin (○), but not their activation by streptokinase (●). Results were obtained with four plasmas. Hexadimethrine was added to plasma from −40 to −30 min, kaolin (20 mg/ml.) at −4 min and streptokinase (1,000 U/ml.) at −1 min. At zero time, the test plasmas were incubated in 0.02 M-*p*-tosyl-L-arginine methyl ester hydrochloride and 0.2 M-tris-(hydroxymethyl)aminomethane buffer, *p*H 8.0, at 37° C. The final dilution of plasma was six-fold. Abscissa: hexadimethrine concentration (mole × 10^{-4}, based on a molecular weight of 6,000). Ordinate: percentage inhibition of *p*-tosyl-L-arginine methyl ester hydrolysis measured from 0 to 60 min.

competed with hexadimethrine for enzymic sites. Table 1 also shows that previous incubation with *p*-tosyl-L-arginine methyl ester did not interfere with the activating effect of kaolin. These findings suport the report by Ratnoff, Davie & Mallett (1961) that Hageman factor does not accept *p*-tosyl-L-arginine methyl ester as a substrate.

There was no evidence that the effect of hexadimethrine on factors splitting *p*-tosyl-L-arginine methyl ester in plasma was progressive. Inhibition by any hexadimethrine concentration remained unchanged when the duration of its incubation with plasma before activation by kaolin was varied at 37° C between 1 and 120 min.

Neutralization of hexadimethrine by heparin. Hexadimethrine inhibited activation by kaolin of human and guinea-pig serum, and of plasma containing anticoagulant concentrations of oxalate, citrate or heparin. Attempts to neutralize fully hexadimethrine were complicated by the fact that in higher concentrations heparin itself induced in plasma some splitting of *p*-tosyl-L-arginine methyl ester. Nevertheless it was clear that "equivalent" concentrations of heparin neutralized inhibitory concentrations of hexadimethrine. Thus, hexadimethrine seemed to adhere less firmly to kaolin than to glass. The effectiveness of heparin was not doubled when it was added to plasma immediately after, instead of together with hexadimethrine. This response was different from the interaction of the two compounds found in plasma thromboplastin generation tests (Shanberge, Regan, Talarico & Busiek, 1961).

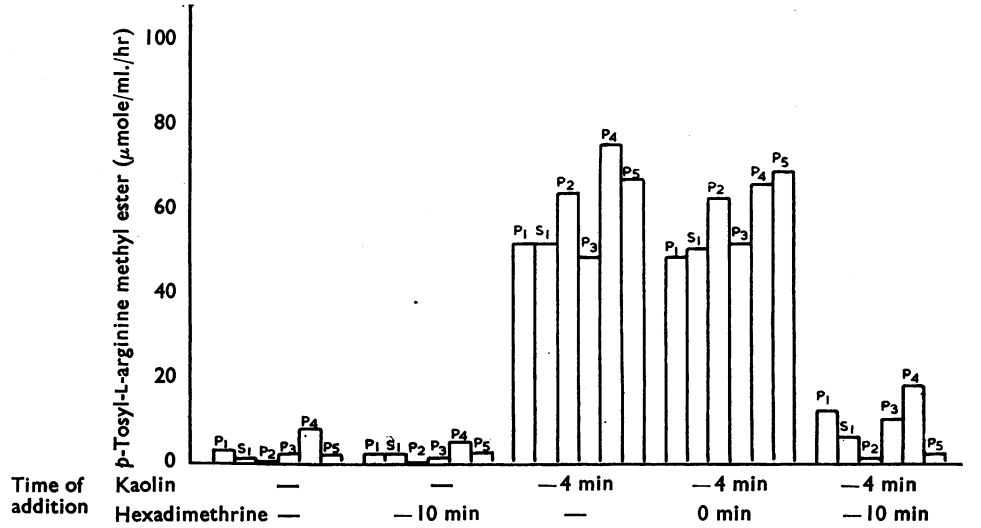

Fig. 8. Inhibition by hexadimethrine added to plasma before kaolin of *p*-tosyl-L-arginine methyl ester hydrolysis, and negligible effect when added after kaolin. Plasma or serum was incubated at zero time with 0.02 M-*p*-tosyl-L-arginine methyl ester hydrochloride and 0.2 M-tris-(hydroxymethyl)aminomethane buffer, *p*H 8.0, at 37° C. The final dilution of plasma was six-fold. The groups of columns show the amounts of *p*-tosyl-L-arginine methyl ester split over 0 to 60 min by five plasmas (P_1 to P_5) and by the serum (S_1) of P_1, under the influence of kaolin (20 mg/ml.) and hexadimethrine (1 mg/ml.) added to the plasmas at the specified times.

TABLE 1

LACK OF INHIBITION BY *p*-TOSYL-L-ARGININE METHYL ESTER OF THE ACTIVATION OF PLASMA BY KAOLIN OR OF THE INHIBITION OF THIS ACTIVATION BY HEXADIMETHRINE

p-Tosyl-L-arginine methyl ester (TAMe) hydrolysis was measured at 0 to 60 min as illustrated in Fig. 7. The concentration of kaolin was 20 mg/ml., and of hexamethrine was 0.5 mg/ml.

Incubation with plasma		Hydrolysis of TAMe
Time (min)	Addition	(μmole/ml. plasma/hr)
0	TAMe	1·5
−20	TAMe	0
−4	Kaolin	
0	TAMe	63·0
−20	TAMe	
−4	Kaolin	68·0
−4	Kaolin	
0	Hexadimethrine, TAMe	61·5
−20	TAMe	
−4	Kaolin	
0	Hexadimethrine	71·0
−10	Hexadimethrine	
−4	Kaolin	
0	TAMe	8·0
−20	TAMe	
−10	Hexadimethrine	
−4	Kaolin	0

Effects of hexadimethrine on fibrinolysis

Exposure to kaolin promotes fibrinolytic activities in plasma (Soulier & Prou-Wartelle, 1959). The influence of hexadimethrine on the action of kaolin was studied using Iatridis & Ferguson's (1962) method with some modifications (Fig. 9). The rapid development of fibrinolytic activity which occurred when the suspended euglobulin fraction is incubated with kaolin at 37° C (Fig. 9, curve 3) was inhibited by previous mixing of the test plasma with hexadimethrine (Fig. 9, curves 4, 5 and 7). The " spontaneous " activation of fibrinolysis in siliconed vessels was also slower in suspensions from plasma samples containing hexadimethrine than in control samples (Fig. 9, curves 1 and 2). Fibrinolysis was not significantly inhibited when corresponding amounts of hexadimethrine were added to the euglobulin suspensions at the end of the period of activation by kaolin (Fig. 9, curves 6 and 8).

Hexadimethrine did not interfere with the activation of the fibrinolytic system by streptokinase or with the resulting activity. In concentrations above 100 μg/ml., hexadimethrine even accelerated spontaneous or streptokinase-induced dissolution of clots. This was seen with whole plasma clots (containing both fibrinolytic factors and inhibitors) and with clots free of inhibitors (made of human plasma euglobulin with bovine fibrinogen). The faster lysis of these clots was possibly due to the anticoagulant effects of hexadimethrine (Shanberge *et al.*, 1961) which may have interfered with the formation of the clots. This possibility was excluded in experiments on bovine fibrin plates. Tests on unheated plates measure mainly the activity of plasminogen activator. Hexadimethrine inhibited neither the formation of plasminogen activator by streptokinase nor its activity. The tests on heated plates which are specific for measuring plasmin showed that it is also not inhibited by hexadimethrine.

Effects of hexadimethrine on capillary permeability

In guinea-pigs, intracutaneous injections of kaolin suspensions (2.5 to 5 mg/ml.) produced a local increase of capillary permeability. The effect appeared to be due to activated proteolytic factors, since it was inhibited by soya bean trypsin inhibitor (0.05 to 1 mg/ml.) but not by mepyramine (50 μg/ml.). Presence in the suspension of hexadimethrine (50 to 500 μg/ml.) prevented blueing. Kaolin which had previously been immersed in hexadimethrine and then washed and resuspended was also less active. Hexadimethrine (500 μg/ml.) did not reduce the blueing produced by purified guinea-pig permeability factor.

The effect of kaolin suspensions was also depressed when hexadimethrine was administered systemically. First, the intracutaneous tests were made, using the left flank. After 30 min, when the blueing reaction was completed, hexadimethrine (1 to 5 mg/kg) was given either in a single intravenous injection or in a priming dose followed by a continuous infusion. After 10 min from the initial injection of hexadimethrine, the intracutaneous tests were performed on the right flank so that the positions of each injection on the two flanks were symmetrical. Hexadimethrine (1 to 2 mg/kg) clearly depressed the effect of kaolin, but not that of permeability factor. Hexadimethrine (3 to 5 mg/kg) abolished the responses both to kaolin and to permeability factor. Local and systemic administration of hexa-

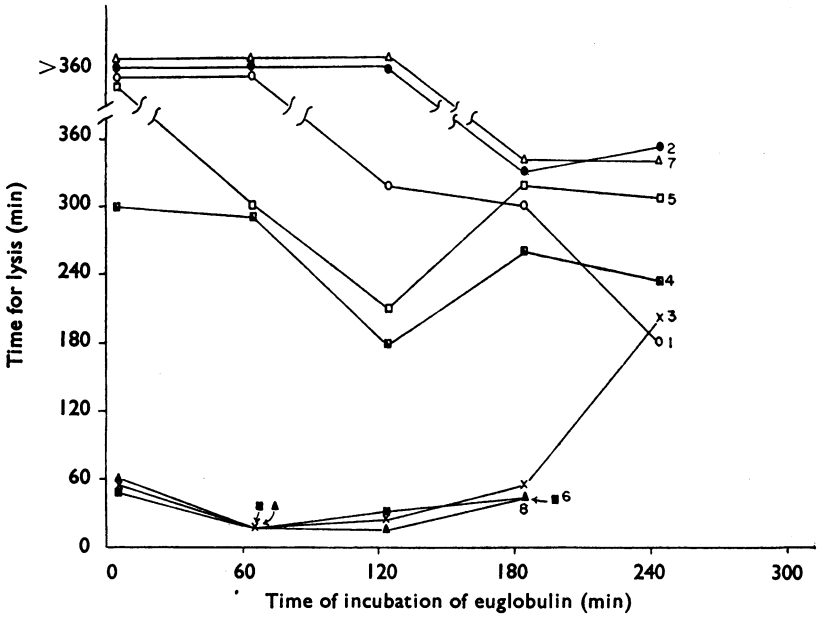

Fig. 9. Inhibition by hexadimethrine of spontaneous and kaolin-induced activation of fibrinolysis in euglobulin suspensions. Suspensions of the euglobulin fraction were obtained at zero time by diluting plasma twenty times at *p*H 5.2 in acetic acid. The suspensions were incubated at 37° C and the development of fibrinolytic activity was observed by centrifuging 3 ml. aliquots at intervals (abscissa); the precipitated euglobulins were dissolved in 0.15 ml. of Michaelis-saline buffer, *p*H 7.4, and mixed with 0.15 ml. of bovine fibrinogen solution (0.3%) and 0.15 ml. of thrombin solution (3 U total). The time from formation to dissolution of clots at 37° C was recorded (ordinate). Hexadimethrine and kaolin were applied as follows:

Graph		Hexadimethrine (μg/ml. plasma) added at		Kaolin (mg./ml. plasma) added at
No.	Symbol	−20 min	End of activation	−5 min
1	○—○	0	0	0
2	●—●	50	0	0
3	×—×	0	0	1·5
4	◪—◪	1	0	1·5
5	□—□	10	—	1·5
6	■—■	0	10	1·5
7	△—△	50	—	1·5
8	▲—▲	0	50	1·5

Note that kaolin-induced activity (3, was inhibited when hexadimethrine was added before kaolin (4, 5 and 7); addition after kaolin has no effect (6 and 8).

dimethrine increased the incidence and extent of bleeding at the sites of intracutaneous injections.

In rats, hexadimethrine (50 to 100 μg/ml., intradermally) itself increased capillary permeability. On the other hand, kaolin had only a negligible effect. No evidence could be obtained that addition of hexadimethrine to the test solutions reduced the blueing produced by dextran, ovomucoid trypsin inhibitor, purified human and

guinea-pig permeability factors, human plasma kallikrein and tetrahydrofurfuryl alcohol.

DISCUSSION

This study suggests that the inhibitory effect of hexadimethrine on intrinsic plasma kinin formation consists mainly of two components: (1) an inhibition of active Hageman factor; and (2) an action on foreign surfaces which reduces their capacity to activate Hageman factor.

Active Hageman factor was inhibited by hexadimethrine in concentrations of 20 to 200 μg/ml. and was thus at least ten-times more sensitive than was the plasma enzyme which releases kinin from substrate. In studies on capillary permeability, Ratnoff & Miles (1962) observed a similar sensitivity of Hageman factor to hexadimethrine.

Although the purified substances Thrombolysin, permeability factor, and kinin-forming substance induced intrinsic plasma kinin formation in the same manner as did purified Hageman factor, that is, by activating another enzyme in plasma, they were not clearly inhibited by hexadimethrine in concentrations up to 1 mg/ml. Moerover, they differed from Hageman factor by their failure to coat glass surfaces with an activating agent. It is therefore possible that plasma contains, besides Hageman factor, other activators of the kinin-forming enzyme. Such a function has recently been attributed to plasmin and to permeability factor:

1. In previous work, Eisen (1961, 1963b) found that the activation of kinin-forming enzyme in plasma by Thrombolysin could not be correlated with the proteolytic potency of Thrombolysin. The results suggested the presence in Thrombolysin of a contaminant acting like Hageman factor. Vogt (1963) has recently postulated that plasmin itself activates kinin-forming enzyme (kallikrein). The present study does not exclude this possibility since, in addition to Thrombolysin, all other purified preparations contained some fibrinolytic activity. Human permeability factor and kinin-forming substance lysed both unheated and heated fibrin. Streptokinase enhanced these activities and also induced some fibrinolytic activity in purified human plasma kallikrein. Ratnoff & Davie (1962) have reported that their preparation of Hageman factor also contains some plasminogen.

2. Mason & Miles (1962) have presented evidence that permeability factor acts not by forming kinin but by activating kinin-forming enzyme (kallikrein). Some of the reported chemical and functional properties of permeability factor suggest that it differs both from plasmin and from Hageman factor.

The available evidence that Hageman factor is an activator of the kinin-forming enzyme is convincing, but the role of other factors in this activation needs further study.

Since the activating properties of glass and other silicates have been attributed to negative charges on their surface, it seems plausible that hexadimethrine inactivates these surfaces by virtue of its positive charges. However, the failure of other positively charged compounds to inactivate glass suggests that some specific molecular configuration is required. Since glass was inactivated by each of the three antiheparin agents tested, it is likely that its surface shares some physicochemical

property with heparin. Armstrong & Stewart (1962) suggested that both glass and heparin induce plasma kinin formation " by sequestering off the natural inhibitor in plasma." However, no unequivocal evidence has yet been obtained that glass adsorbs an inhibitor from normal plasma, whereas large amounts of adsorbed active Hageman factor and of kinin-forming enzyme can readily be demonstrated (Waaler, 1959 ; Margolis, 1960 ; Ratnoff *et al.*, 1961 ; Eisen, 1963a). Whatever the mechanism of the inactivation of glass, its affinity for hexadimethrine must be very high, since the latter was effective even when mixed and neutralized with a tenfold excess of heparin.

The finding that formation of kinin induced in plasma by dilution, acid, acetone and ε-aminocaproic acid is reduced in vessels treated with hexadimethrine suggests that surface plays an important role even in these modes of activation. It is probable that even the most " inert " foreign surfaces available cause some activation of Hageman factor ; many soluble agents which induce intrinsic plasma kinin formation may merely suppress the inhibitors of this surface activation and of its sequels. The results described suggest that glass treated with hexadimethrine has at least as inert a surface as has silicone or polyethylene.

The potency of the plasma enzyme which acts directly on kinin-yielding substrate was only occasionally reduced by the highest concentration of hexadimethrine (1 mg/ml.). This concentration tends to denature plasma proteins (B. Mason, personal communication), which may account for the occasional " inhibition."

The studies on *p*-tosyl-L-arginine methyl ester hydrolysis and on fibrinolysis also showed that hexadimethrine inhibits the activation of the enzymes, and not their activity. The evidence that kaolin accelerates fibrinolysis by activating Hageman factor has been confirmed and analysed in considerable detail by Iatridis & Ferguson (1961, 1962). The inhibition by hexadimethrine of the " spontaneous " development of fibrinolysis suggests that this development is also promoted by the siliconed vessel walls which produce a slow, mild activation of Hageman factor.

In the experiments using *p*-tosyl-L-arginine methyl ester, there was no sign that active Hageman factor was inhibited, since hexadimethrine added after kaolin, but together with *p*-tosyl-L-arginine methyl ester, had practically no effect. It is possible that, in the conditions of these experiments, active Hageman factor had completed the activation of the enzyme which splits *p*-tosyl-L-arginine methyl ester at the time when hexadimethrine and *p*-tosyl-L-arginine methyl ester were added.

Hexadimethrine did not inhibit the activation by streptokinase of digestion of *p*-tosyl-L-arginine methyl ester or fibrin. This result supports the evidence that the plasma factor activated by streptokinase is not Hageman factor (Iatridis & Ferguson, 1961).

The inactivation of kaolin by hexadimethrine was also evident in the studies on capillary permeability. This suggests that an important part of the *in vivo* reaction to kaolin, and possibly to other foreign bodies, may be due to the activation of Hageman factor and the resulting enzymic processes.

A better understanding of the physico-chemical properties which enable a foreign surface to activate Hageman factor will help to reveal whether *in vivo* some chemical

compounds or pathologically altered cells and tissues may constitute similar surfaces. Such an effect for long-chain saturated fatty acids has been claimed by Connor, Hoak & Warner (1963) and Margolis (1962), but denied by Botti & Ratnoff (1963).

If activation of Hageman factor by surfaces does occur *in vivo*, compounds like hexadimethrine may prove useful in preventing this process. For such an application it may be of particular interest that this effect of hexadimethrine is in some cases not abolished by concentrations of heparin which neutralize all its other known effects.

The use of glassware treated with hexadimethrine in the preparation of therapeutic plasma fractions might reduce the formation of kinins in the recipient when these fractions are transfused. Clinical reactions to human antihaemophilic globulin concentrate have been attributed to contamination with precursors of kinin-forming factors and with substrate (Mackay, Maycock & Combridge, 1962). In one batch of this preparation a potent active factor which could induce plasma kinin formation was also found (Eisen, unpublished).

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REFERENCES

ALKJAERSIG, N., FLETCHER, A. P. & SHERRY, S. (1959). The mechanism of clot dissolution by plasmin. *J. clin. Invest.*, **38**, 1086–1095.

ARMSTRONG, D. A. J. & STEWART, J. W. (1962). Anti-heparin agents as inhibitors of plasma kinin formation. *Nature (Lond.)*, **194**, 689.

ASTRUP, T. & MÜLLERTZ, S. (1952). The fibrin plate method. *Arch. Biochem.*, **40**, 346–351.

BIGGS, R. & MACFARLANE, R. G. (1957). *Human Blood Coagulation and its Disorders*, 2nd ed., p. 387. Oxford: Blackwell Scientific Publications.

BOTTI, R. E. & RATNOFF, O. D. (1963). The clot-promoting effect of soaps of long-chain saturated fatty acids. *J. clin. Invest.*, **42**, 1569–1577.

CONNOR, W. E., HOAK, J. C. & WARNER, E. D. (1963). Massive thrombosis produced by fatty acid infusion. *J. clin. Invest.*, **42**, 860–866.

EISEN, V. (1961). *Modes of Formation of Human Plasma Kinin.* Ph.D. Thesis, University of London.

EISEN, V. (1963a). Observations on intrinsic kinin-forming factors in human plasma: the effect of acid, acetone, chloroform, heat and euglobulin separation. *J. Physiol. (Lond.)*, **166**, 496–513.

EISEN, V. (1963b). Kinin formation and fibrinolysis in human plasma. *J. Physiol. (Lond.)*, **166**, 514–529.

EISEN, V. & KEELE, C. A. (1960). Intrinsic kinin forming factors in human plasma. *J. Physiol. (Lond.)*, **150**, 21–22*P*.

IATRIDIS, S. G. & FERGUSON, J. H. (1961). Effect of surface and Hageman factor on the endogenous or spontaneous activation of the fibrinolytic system. *Thrombos. Diathes. haemorrh. (Stuttg.)*, **6**, 411–423.

IATRIDIS, S. G. & FERGUSON, J. H. (1962). Active Hageman factor: a plasma lysokinase of the human fibrinolytic system. *J. clin. Invest.*, **41**, 1277–1287.

KIMURA, E. T., YOUNG, P. R., STEIN, R. J. & RICHARDS, R. K. (1959). Some pharmacologic characteristics of hexadimethrine bromide (Polybrene). *Toxicol. appl. Pharmacol.*, **1**, 185–202.

LASSEN, M. (1958). The esterase activity of the fibrinolytic system. *Biochem. J.*, **69**, 360–366.

MACKAY, M., MAYCOCK, W. D'A. & COMBRIDGE, B. S. (1962). Activation of endogenous plasma proteolytic enzymes with the consequent production of pharmacologically active polypeptides. *Nature (Lond.)*, **195**, 1206–1207.

MARGOLIS, J. (1958). Activation of plasma by contact with glass: evidence for a common reaction which releases plasma kinin and initiates coagulation. *J. Physiol. (Lond.)*, **144**, 1–22.

MARGOLIS, J. (1960). The mode of action of Hageman factor in the release of plasma kinin. *J. Physiol. (Lond.)*, **151**, 238–252.

MARGOLIS, J. (1962). Activation of Hageman factor by saturated fatty acids. *Aust. J. exp. Biol. med. Sci.*, **40**, 505–514.

MARGOLIS, J. (1963). The interrelationship of coagulation of plasma and release of peptides. *Ann. N.Y. Acad. Sci.*, **104**, 133–145.

MASON, B. & MILES, A. A. (1962). Globulin permeability factors without kininogenase activity. *Nature (Lond.)*, **196**, 587–588.

MILES, A. A. & WILHELM, D. L. (1955). Enzyme-like globulins from serum reproducing the vascular phenomena of inflammation. I. An activable permeability factor and its inhibitor in guinea-pig serum. *Brit. J. exp. Path.*, **36**, 71–81.

MILSTONE, H. (1941). A factor in normal human blood which participates in streptococcal fibrinolysis. *J. Immunol.*, **42**, 109–116.

PATON, W. D. M. (1957). A pendulum auxotonic lever. *J. Physiol. (Lond.)*, **137**, 35–36*P*.

PRESTON, F. W. (1952). The antiheparin effect of Polybrene. *J. Lab. clin. Med.*, **40**, 927–928.

RATNOFF, O. D. & COLOPY, J. E. (1955). A familial haemorrhagic trait associated with deficiency of a clot-forming fraction of plasma. *J. clin. Invest.*, **34**, 601–613.

RATNOFF, O. D. & DAVIE, E. W. (1962). The purification of activated Hageman factor (activated factor XII). *Biochemistry*, **1**, 967–975.

RATNOFF, O. D., DAVIE, E. W. & MALLETT, D. L. (1961). Studies on the action of Hageman factor: evidence that activated Hageman factor in turn activates plasma thromboplastin antecedent. *J. clin. Invest.*, **40**, 803–819.

RATNOFF, O. D. & MILES, A. A. (1962). Activation of a vascular permeability-increasing factor in human plasma incubated with purified activated Hageman factor. *J. Lab. clin. Med.*, **60**, 1009.

ROBERTS, P. S. (1958). Measurement of the rate of plasmin action on synthetic substrates. *J. biol. Chem.*, **232**, 285–291.

SHANBERGE, J. N., REGAN, E. E., TALARICO, L. & BUSIEK, B. (1961). The effect of Polybrene on blood coagulation *in vitro*. *J. Lab. clin. Med.*, **58**, 23–33.

SOULIER, J. P. & PROU-WARTELLE, O. (1959). Nouvelles données sur les facteurs Hageman et P.T.A., et sur le " contact." *Rev. franc. Etud. clin. biol.*, **4**, 932–938.

VOGT, W. (1963). Kinin formation by plasmin mediated by activation of kallikrein. *J. Physiol. (Lond.)*, **169**, 45–46*P*.

WAALER, B. A. (1959). Contact activation in the intrinsic blood clotting system. *Scand. J. clin. Lab. Invest.*, suppl. 37, 1–133.

WEBSTER, M. E. & PIERCE, J. V. (1960). Studies on plasma kallikrein and its relationship to plasmin. *J. Pharmacol. exp. Ther.*, **130**, 484–491.