J. Physiol. (1958) 140, 285-300

FORMATION OF PLASMA KININS BY PLASMIN

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(*Received* 16 *September* 1957)

Vasodilator polypeptides appear to play some part in the physiology and pathology of vascular control, so that the determination of the conditions under which they occur in the circulation, and an understanding of the mechanism of their formation, are required for a general understanding of the control of peripheral blood vessels. The present experiments describe the formation by plasmin of vasodilator polypeptides, plasma kinins, which is a name applied to bradykinin-like polypeptides derived from plasma proteins. The term plasma kinin and its definition was suggested in a discussion between J. H. Gaddum, S. M. Hilton, C. A. Keele, M. Schachter and myself at the meeting of the British Pharmacological Society at Oxford in June 1957. Our present knowledge about vasodilator polypeptides stems mainly from two groups of workers, Frey, Kraut and Werle in Germany, and Rocha e Silva and Beraldo and their colleagues in Brazil. Frey, Kraut & Werle (1950), isolated, in a crude form, from several tissues and body fluids, a material which caused a fall in blood pressure and had some of the characteristics of an enzyme. Although it is not certain if each of these materials contain the same active principle they have all been given the name of kallikrein. In 1937 Werle, Götze & Keppler made the observation that when kallikrein came into contact with blood proteins a second substance was formed, which was not a protein like kallikrein but a smaller molecule. However, it was not fully appreciated that this second substance was the agent principally responsible for the actions of kallikrein until Rocha e Silva, Beraldo & Rosenfeld (1949) observed that trypsin and the proteolytic enzymes in certain snake venoms, on incubation with plasma proteins, formed a polypeptide which they called bradykinin and which was later found to be indistinguishable from the second substance of the German workers, called kallidin.

It seems likely that there are several proteolytic enzymes capable of splitting one of the plasma proteins, probably an α_2 globulin, in a similar manner, producing a group of closely related polypeptides with powerful smooth

muscle stimulating and vasodilator activity. Substances of this group are referred to as plasma kinins. It seems probable that the plasma kinins, bradykinin and kallidin are in fact mixtures of polypeptides and when using these terms on the following pages this possibility is not meant to be excluded.

Plasmin (fibrinolysin) is a proteolytic enzyme which is found in blood, normally present as an inactive precursor called plasminogen (Christensen & MacLeod, 1945). Recently, Scandinavian workers (Astrup, 1956; Mullertz, 1956) have discovered that extracts of many tissues are capable of activating plasminogen to the active proteolytic enzyme. If plasmin were able to form vasodilator plasma kinins, injured tissues, containing plasminogen activators, would form vasodilator polypeptides and these would then take part in the tissue reaction. Evidence in favour of the view that a smooth muscle stimulating substance is formed by plasmin has already been reported by several authors. Beraldo (1950) and Schachter (1956) have shown that bovine plasmin gives rise to smooth muscle stimulating activity when incubated with plasma proteins and Lewis & Work (1957), as well as Armstrong, Jepson, Keele & Stewart (1957), found the same for human plasmin. In the present experiments it will be shown that the plasma kinin formed by human plasmin is indistinguishable from bradykinin. Further, using proteolytic inhibitors it was found that the activation of plasma when treated with chloroform or when brought into contact with a glass surface results from the activation of plasminogen to plasmin with the subsequent formation of a plasma kinin.

METHODS

In order to test whether plasmin acted upon plasma proteins to form a smooth muscle stimulating substance, samples of the enzyme preparations were incubated with pseudo-globulin solution and the incubated mixture tested on isolated smooth muscle preparations.

The principle in the incubated mixture of plasmin and pseudo-globulin solution responsible for smooth muscle stimulating activity was then extracted, using the same procedure as that employed by Andrade & Rocha e Silva (1957) for the extraction of crude bradykinin from an incubated mixture of snake venom and pseudo-globulin. For this purpose human plasmin 1 g (Merck, Sharpe & Dohme) was incubated with 50 ml. pseudo-globulin solution at 37° C for 20 min. Two volumes of boiling absolute alcohol were added and the mixture boiled for a further 10 min. After centrifugation, the filtrate was evaporated to dryness *in vacuo* at a temperature below 50° C. The residue was taken up in glacial acetic acid and precipitated with 9 volumes of ether. The powder prepared in this way was compared with a standard preparation of bradykinin on the following preparations.

Smooth muscle preparations. The isolated guinea-pig's ileum, rat's uterus and rat's colon were suspended in a 15 ml. organ bath. For the guinea-pig's ileum the bath fluid was Mg-free Tyrode solution containing atropine (10^{-8} g/l.), maintained at 34° C; and for the rat's tissues de Jalon's solution was used at 28° C for the uterus and at 24° C for the colon.

Vasodilatation was examined in the skin of the hind leg supplied by the saphenous artery. Cats were anaesthetized with pentobarbitone sodium (40 mg/kg) and the femoral artery dissected beyond the origin of the saphenous artery, all muscle branches being tied off. A cannula for arterial injection was then inserted into the femoral artery beyond the origin of the saphenous artery. Since all branches of the femoral artery below the inguinal ligament were ligated, sub-

stances in a volume of 0·2–0·3 ml. would be carried by the blood stream into the saphenous artery. The femoral vein was also dissected and its side branches except the saphenous vein were ligated. The femoral vein was then tied distal to the saphenous vein and a cannula inserted into the femoral vein so as to allow the blood from the saphenous vein to pass a photo-electric drop counter after the cat had been given heparin (10 mg/kg). The outflow was recorded with a Gaddum drop timer, and the blood allowed to pass back into the femoral vein.

Estimation of plasmin. The proteolytic activity of the enzyme preparations was determined by (*a*) hydrolysis of casein, measuring the increase in optical density at 275 mμ of the deproteinized reaction mixture as described by Mullertz (1955), (*b*) hydrolysis of benzoylarginine ethyl ester, measuring optical density at 253 mμ, employing the method of Schwert & Takenake (1955). The unit of activity was taken as (*a*) μg of tyrosine liberated in 1 hr at 37° C, (*b*) μg of benzoylarginine liberated in 1 hr at room temperature. The proteolytic activity was expressed as units/mg protein.

Estimation of protein content of plasmin preparations. The protein content was expressed in terms of bovine serum albumin. The protein was precipitated with excess of 1·7M perchloric acid and the precipitate dissolved in 0·1 N-NaOH. The ultra-violet absorption was read at 285 mμ using a Hilger Uvispek spectrophotometer and the protein content estimated from a standard curve for bovine serum albumin.

Materials

Human plasmin. (1) 'Mullertz plasmin' was prepared according to the method of Mullertz (1955), using citrated human plasma obtained from the Edgware transfusion centre. (2) Human plasmin (Merck) was kindly supplied by Merck, Sharpe and Dohme Ltd.

Bovine plasmin was a gift of Parke, Davis Company.

Human plasminogen was prepared by the method of Kline (1953) the final solution being freeze-dried in 2 ml. ampoules and stored at −10° C. Citrated human plasma from the Edgware transfusion centre was used in its preparation.

Streptokinase. Streptokinase-Streptodornase (Burroughs Wellcome).

Trypsin. Crystalline trypsin (Armour Ltd.).

Casein solution (3%) was prepared from goat casein by the method of Mullertz (1955).

Benzoylarginine ethyl ester was kindly prepared by Mr A. Hemmings of the National Institute for Medical Research.

Pseudo-globulin solution was prepared from dog plasma and consisted of the fraction of plasma protein precipitated by $(NH_4)_2SO_4$ 33–46% saturation. This precipitate was dialysed for 48 hr against running tap water and the final solution (one-third of the original volume) freeze-dried in 2 ml. ampoules and stored at −10° C.

Bradykinin was kindly supplied by Dr W. Beraldo.

RESULTS

Formation of a smooth muscle stimulating substance by plasmin

For determining the development of smooth muscle stimulating activity by the action of plasmin, the pseudo-globulin fraction of plasma proteins was used as substrate. Plasma itself was not suitable as substrate, as most samples of plasma contain an inhibitor of plasmin. In the case of human plasma even the pseudo-globulin fraction contains plasmin inhibitor, so that usually pseudo-globulin prepared from dog's plasma was used as substrate. When samples of plasmin prepared by Mullertz's method were incubated with this pseudo-globulin the incubated mixture acquired smooth muscle stimulating activity. The development of this activity in incubated mixtures of human plasmin and

dog pseudo-globulin is shown on the guinea-pig's ileum in Fig. 1. The activity of the incubated mixture was not due to either histamine or acetylcholine, as the contractions were unaffected by the presence of the antagonists mepyramine or atropine. However, the response of the guinea-pig's ileum closely resembled that of the polypeptide bradykinin in being a slow contraction.

The experiment of Fig. 1 also illustrates that as incubation proceeded the smooth muscle stimulating activity of the mixture increased, reaching a maximum after about 20 min incubation at 34° C. More prolonged incubation resulted in the gradual decrease of activity which eventually disappeared altogether. This is exactly what happens when trypsin is incubated with the same substrate to form bradykinin.

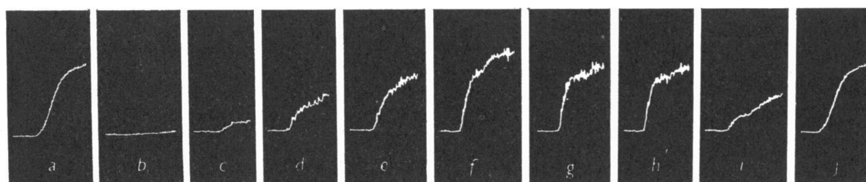

Fig. 1. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*) and (*j*) bradykinin 10 μg. Plasmin 4 mg (*b*) caused no contraction. Mixture of plasmin 4 mg and pseudo-globulin solution 0·2 ml. incubated for 2 min at (*c*), 5 min at (*d*), 10 min at (*e*), 15 min at (*f*), 20 min at (*g*), 25 min at (*h*) and 40 min at (*i*).

The disappearance of the smooth muscle stimulating activity in a mixture of trypsin and pseudo-globulin or of plasmin and pseudo-globulin does not result from the destruction of bradykinin by trypsin or plasmin but from the presence in the pseudo-globulin fraction of a bradykinin-destroying factor. Evidence of this is shown by the fact that if bradykinin itself is incubated with the pseudo-globulin solution its smooth muscle stimulating activity gradually diminishes and eventually disappears.

The striking similarity between the formation and deterioration of the smooth muscle stimulating activity of incubated mixtures of trypsin and pseudo-globulin on the one hand and plasmin and pseudo-globulin on the other suggested that in both instances the smooth muscle stimulating activity might be due to the same plasma kinin—bradykinin.

The human plasmin made by Merck, Sharpe and Dohme Ltd. behaved like Mullertz's plasmin when incubated with pseudo-globulin, producing smooth muscle stimulating activity which increased with time of incubation and reached a maximum after 20 min incubation at 34° C.

Bovine plasmin (Parke, Davis) when incubated with pseudo-globulin under the same conditions did not cause contraction of the smooth muscle preparations. This, however, does not necessarily mean that a plasma kinin was not formed, because even if it were formed it would not have contracted the smooth

muscle since bovine plasmin itself renders these preparations insensitive. For example, the guinea-pig's ileum did not respond to histamine, 5-hydroxytryptamine, substance P or bradykinin for more than 1 hr after 5 mg of bovine plasmin was added to the 15 ml. organ bath for 5 min. In contrast it was observed that both human plasmin and streptokinase potentiated the responses of the guinea-pig's ileum to bradykinin.

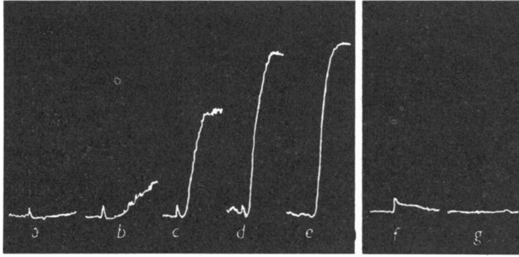

Fig. 2. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. Mixture of plasminogen 0·5 mg, streptokinase 1000 units and pseudo-globulin solution 0·1 ml. incubated for 2 min at (*a*), 5 min at (*b*), 10 min at (*c*), 15 min at (*d*) and 20 min at (*e*). At (*f*) plasminogen and pseudo-globulin solution 0·1 ml. and at (*g*) streptokinase 1000 units and pseudo-globulin solution 0·1 ml., each incubated for 20 min.

The precursor of plasmin, plasminogen, prepared from human plasma, did not give rise to any smooth muscle stimulating activity when incubated with pseudo-globulin solution. But when streptokinase, which activates plasminogen to plasmin, was added to a mixture of plasminogen and pseudo-globulin the mixture caused a contraction of isolated smooth muscle. This is shown in the experiment of Fig. 2 on the guinea-pig's ileum. As in the experiment in which plasmin was incubated with pseudo-globulin, the smooth muscle stimulating activity of an incubated mixture of plasminogen, streptokinase and pseudo-globulin increased with time of incubation and reached a maximum after 20 min incubation. No smooth muscle stimulating activity developed when streptokinase and plasminogen or streptokinase and pseudo-globulin were incubated for 20 min.

Comparison of the plasma kinin formed by plasmin with bradykinin

A detailed comparison was made of the active principle isolated from an incubated mixture of plasmin and pseudo-globulin solution with a standard preparation of bradykinin. The plasma kinin caused the same type of response as bradykinin when added to smooth muscle preparations which respond biphasically to bradykinin. The experiment of Fig. 3 shows the biphasic responses of the isolated rat's colon to the plasma kinin prepared from plasmin and pseudo-globulin, to bradykinin, and to another smooth muscle stimulating polypeptide, substance P.

The response of the guinea-pig's ileum to bradykinin is a slow contraction, preceded by a short latency and followed by a slow relaxation. Fig. 4 illustrates the similarity in the responses of the guinea-pig's ileum to both the plasma kinin and to bradykinin and the different, more rapid, response to substance P. Further, both the plasma kinin and bradykinin cause contraction of the isolated rat's uterus. The responses of this tissue to bradykinin are characterized by the fact that the latency period is directly proportional to the amount of substance added. Such a latency also occurred when the plasma kinin formed by plasmin was added to the organ bath containing a rat's uterus.

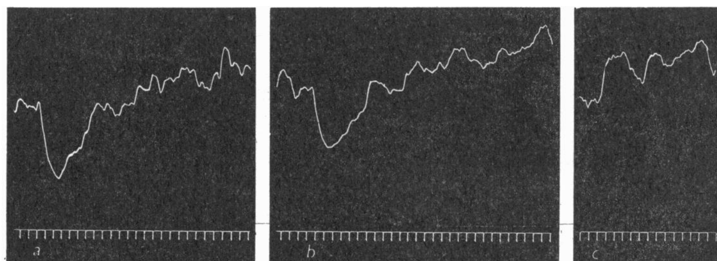

Fig. 3. Rat's colon suspended in 15 ml. of de Jalon's solution. Time marker, 30 sec. At (*a*) powder prepared from incubated mixture of plasmin and pseudo-globulin, 4 mg; at (*b*) bradykinin 300 μg; at (*c*) substance P 100 μg.

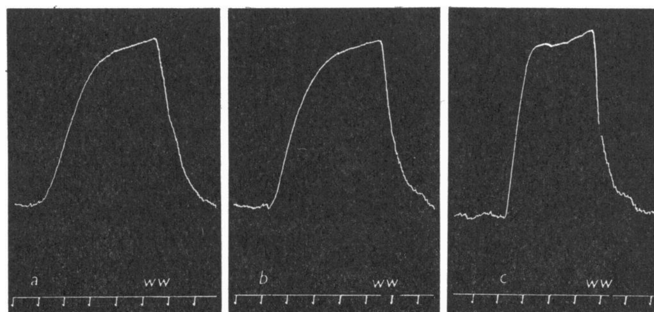

Fig. 4. Guinea-pig's ileum suspended in 15 ml. of Mg-free Tyrode solution. Time marker, 30 sec. At (*a*) powder prepared from incubated mixture of plasmin and pseudo-globulin, 0·5 mg; at (*b*) bradykinin 15 μg; at (*c*) substance P 150 μg. *W* indicates that the tissue was washed by allowing the bath to overflow.

When injected arterially into the skin of a cat's hind limb both substances produce a pronounced vasodilatation. The experiment of Fig. 5 illustrates this and also shows the response to substance P under the same conditions.

Quantitative comparison

Although qualitatively the actions of the plasma kinin prepared from plasmin and pseudo-globulin were similar to those of bradykinin in all tests, a quantitative comparison on several tissues is desirable in helping to establish the

identity of the plasma kinin as bradykinin. In order to show the value of this quantitative test more clearly, the sensitivity of four tissues to the plasma kinin, to bradykinin and for comparison, to substance P as well, was estimated and the results are shown in Table 1. This table gives the amounts of bradykinin and substance P which have equal activity with 1 mg of the plasma

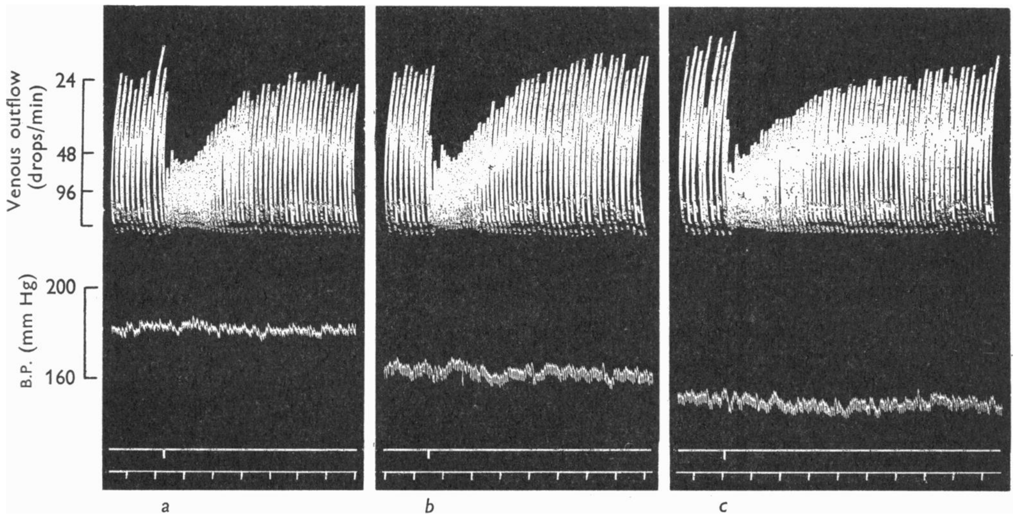

Fig. 5. Cat 2·7 kg, anaesthetized with pentobarbital 40 mg/kg. Upper tracing record of venous outflow from the skin of the hind limb. Lower tracing, arterial blood pressure. Arterial injection of bradykinin 80 μg at (*a*), of the powder made from plasmin and pseudo-globulin 0·8 mg at (*b*), and of substance P 100 μg at (*c*). Time marker, 30 sec.

TABLE 1. Amounts of bradykinin and substance P of equal activity with 1 mg of the powder, prepared from an incubated mixture of plasmin and pseudo-globulin

Tissue	Bradykinin (μg)	Substance P (μg)
Guinea-pig's ileum	35	377
Rat's uterus	37	3750
Rat's colon	53	11
Vasodilatation in cat's skin	60	150

kinin from plasmin when all three substances are tested under the same conditions on the rat's uterus, the rat's colon, the guinea-pig's ileum and the blood flow through the skin of the cat's hind limb. Each figure is the average of three experiments. The results show clearly that within the limits of experimental error the sensitivity of these preparations to the plasma kinin and bradykinin varies in an identical manner, whereas in the case of substance P there are large discrepancies.

Stability

The plasma kinin prepared from plasmin and pseudo-globulin solution behaved like bradykinin when subjected to simple chemical tests. Activity was unaffected by boiling for 1 hr in neutral or acid solution (N-HCl), but disappeared after boiling for a few minutes in 0·1 N-NaOH. In addition, both plasma kinins were destroyed after 1 hr incubation with 100 μg chymotrypsin, but not when incubated with 100 μg of trypsin for 1 hr.

Plasma kinin-forming activity of the plasmin preparations

In order to examine quantitatively the plasma kinin-forming activity of the plasmin preparations and of activated plasminogen, they were incubated with excess of substrate at 34° C for 20 min, the mixtures then assayed against a standard bradykinin on the isolated guinea-pig's ileum, and the plasma kinin-forming activity was expressed as micrograms of bradykinin formed per milligram of protein. It was found with this method that the plasmin preparations and activated plasminogen from human plasma were far less active than crystalline trypsin, as is shown in Table 2. The estimated amounts of bradykinin in the incubated mixtures err on the low side because of the presence in the pseudo-globulin fraction of an agent which destroys bradykinin.

TABLE 2

	Proteolytic activity (units/mg protein)	Esterolytic activity (units/mg protein)	Plasma-kinin-forming activity (μg/mg protein)
Crystalline trypsin	73,400	440,400	268
Bovine plasmin (Parke, Davis)	126	815	—
Human plasmin (Merck)	232	1,730	24
Human plasmin (Mullertz)	185	1,250	17
Human plasminogen + streptokinase	617	7,590	40

Proteolytic action

Although the plasmin and activated plasminogen preparations were less active when assayed by the non-specific hydrolysis of casein than by the more specific hydrolysis of synthetic benzoylarginine ethyl ester, the proteolytic and esterolytic activities of the enzyme preparation relative to those of crystalline trypsin run almost parallel. When human plasminogen was tested for proteolytic and esterolytic activity it was found to be almost inactive. However, when streptokinase 500 μ/ml. was added to the mixture of plasminogen and substrate, proteolytic and esterolytic activities developed.

It can be seen from Table 2 that trypsin, which is most active as a proteolytic enzyme, is also most active as a bradykinin-forming enzyme, and similarly for the less active human plasmin preparations the proteolytic and plasma kinin-forming activity run parallel. For bovine plasmin it was not

possible to determine its plasma kinin-forming activity because of its inhibitory action on smooth muscle. It is interesting that the ratio between the proteolytic activities of the human plasmins and of crystalline trypsin is considerably higher than the ratio between the plasma kinin-forming activities of the plasmins and of trypsin. This suggests that, given a preparation of plasmin with a proteolytic activity equivalent to crystalline trypsin, its plasma kinin-forming activity would be considerably greater than that of crystalline trypsin.

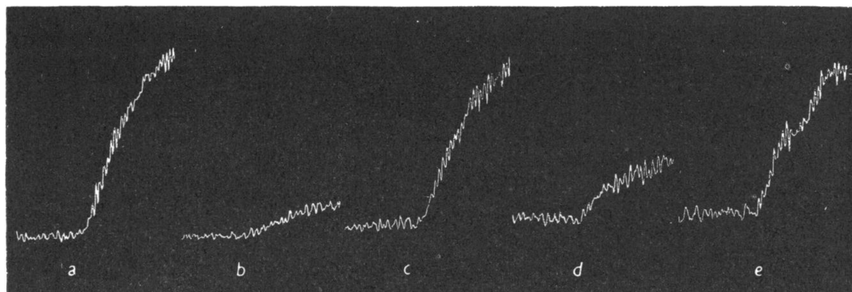

Fig. 6. Guinea-pig's ileum suspended in 15 ml. of Mg-free Tyrode solution. At (*a*), (*c*) and (*e*) plasmin (Merck) 1 mg and pseudo-globulin solution 0·1 ml., incubated for 20 min at 34° C. At (*b*) soya bean trypsin inhibitor 100 μg and at (*d*) antiplasmin 3 mg were added to the mixture of plasmin and pseudo-globulin before incubation.

Inhibition of the plasma kinin-forming activity of plasmin by antiplasmin

Plasma is known to contain a proteolytic inhibitor (Hahn, 1897) which has been isolated from plasma and purified and is known as antiplasmin (Loomis, Ryder & George, 1949). Antiplasmin as well as another inhibitor of proteolytic enzymes, soya bean trypsin inhibitor, was found to inhibit the formation of plasma kinins when plasmin is incubated with pseudo-globulin. The result of such an experiment is illustrated in Fig. 6, which shows the response of the guinea-pig ileum to incubated mixtures of plasmin and pseudo-globulin alone (*a*, *c* and *e*) and in the presence of soya bean trypsin inhibitor (at *b*) and of antiplasmin (at *d*). Both antiplasmin and soya bean trypsin inhibitor also inhibited the formation of the smooth muscle stimulating activity in an incubated mixture of plasminogen, streptokinase and pseudo-globulin.

Plasma kinin-forming activity of plasma

Whole blood or plasma usually contains sufficient of the inhibitors of plasmin and of plasminogen activator to hold the proteolytic system in check. Under certain conditions the equilibrium in this system can be upset and the proteolytic activity of plasma then increases. For instance, plasma treated with chloroform develops proteolytic activity (Christensen, 1946) and exhibits

smooth muscle stimulating activity which untreated plasma does not possess. However, when excess antiplasmin is added to plasma before it is treated with chloroform it does not develop this smooth muscle stimulating activity. The experiment of Fig. 7 illustrates the smooth muscle stimulating activity which develops in plasma treated with chloroform, but which does not occur when an excess of antiplasmin has first been added to the plasma.

Smooth muscle stimulating activity also appears when plasma comes into contact with glass (Armstrong *et al.* 1957). It seems likely that contact with a glass surface might activate the precursor of plasmin, particularly since Rapaport, Aas & Owren (1955) have shown several enzymes involved in blood clotting to be activated in this way. If this were so, it would be expected that the activation would be inhibited by antiplasmin. In fact, the smooth muscle

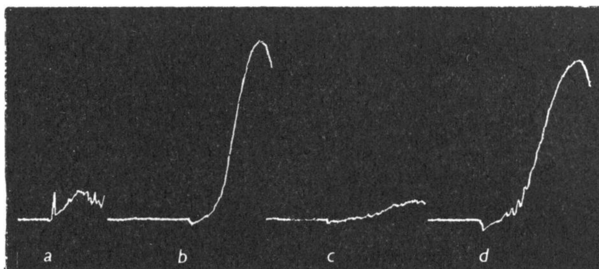

Fig. 7. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*) human plasma 0·5 ml., at (*b*) and (*d*) human plasma 0·5 ml. shaken with chloroform 0·02 ml. and at (*c*) a mixture of human plasma 0·5 ml. and antiplasmin 1·5 mg shaken with chloroform 0·02 ml.

stimulating activity which develops when plasma is transferred from a siliconed glass vessel to an unsiliconed vessel was readily inhibited by addition of antiplasmin. This is illustrated in the experiment of Fig. 8. At *a* and *b* are shown the small contractions of the guinea-pig ileum following addition of 0·3 ml. of freshly drawn human plasma which had been standing in a siliconed glass vessel for 1 and 5 min respectively, and at *c* and *d* are the stronger responses to 0·3 ml. of the same sample of plasma after standing in an unsiliconed glass vessel for 1 and 5 min respectively. When 0·1 ml. of saline containing 300 μg of antiplasmin was present in the unsiliconed glass vessel before allowing the plasma to stand in it then the smooth muscle stimulating activity did not appear, as shown at *e* and *f*. The glass activation of plasma is also inhibited by soya bean trypsin inhibitor (Armstrong *et al.* 1957).

Schachter (1956) showed that plasma diluted with physiological saline developed smooth muscle stimulating activity. Dausset, Bergerot-Blondel & Colin (1956), who found that plasma also develops proteolytic activity on dilution, suggested that this proteolytic activity was due to an activation of plasmin. This suggestion is not borne out by the following observations. When

freshly drawn human plasma kept in a siliconed vessel was diluted 1 to 10 with physiological saline or with Tyrode solution, it developed smooth muscle stimulating activity in 15 min at 34° C, as shown in Fig. 9. The same occurred when 1 or 3 mg/ml. of antiplasmin had been added to the plasma (Fig. 9*c*, *e*).

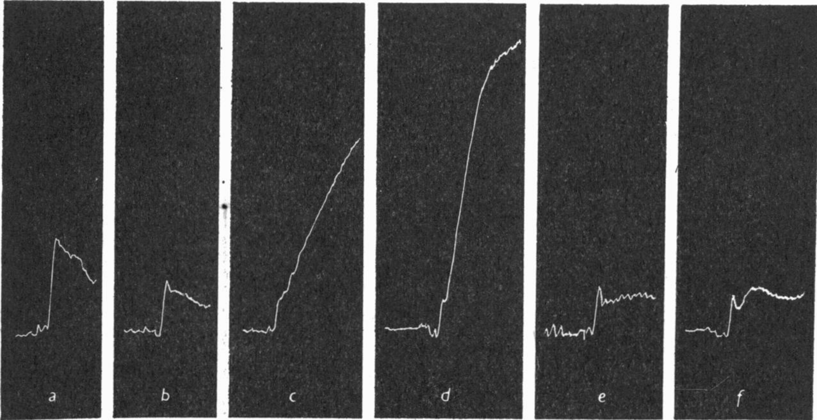

Fig. 8. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*) and (*b*) freshly drawn human plasma 0·3 ml. allowed to stand in a siliconed glass vessel for 1 min and 5 min respectively. At (*c*) and (*d*) 0·3 ml. portions from the same sample of plasma, and at (*e*) and (*f*) plasma 0·3 ml. plus antiplasmin 300 μg, allowed to stand in an unsiliconed glass vessel; (*c*), (*e*) for 1 min and (*d*), (*f*) for 5 min.

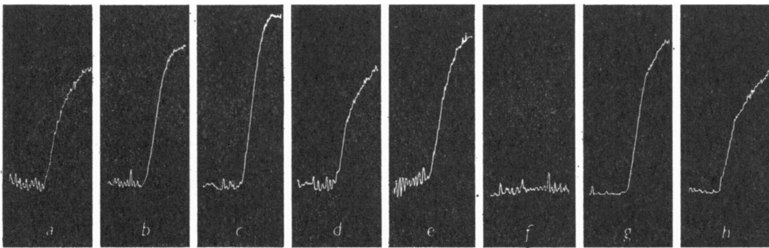

Fig. 9. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*), (*d*) and (*h*) bradykinin 5 μg. Human plasma 0·1 ml. stored in a siliconed vessel diluted 1:10 with Tyrode solution at (*b*) and (*g*). The same plasma 0·1 ml. diluted 1:10 with Tyrode solution containing 1 mg antiplasmin at (*c*), 3 mg antiplasmin at (*e*) and 100 μg soya bean trypsin inhibitor at (*f*).

On the other hand, the addition of soya bean trypsin inhibitor 100 μg/ml. to the plasma completely inhibited the development of the activity (Fig. 9 *f*) as was shown already by Schachter (1956). The concentration of antiplasmin could not be increased above 3 mg/ml. plasma as high doses of antiplasmin caused depression of the smooth muscle.

Miles & Wilhelm (1955) and Wilhelm, Miles & Mackay (1955) have found that an α_2 globulin from guinea-pig serum, which they call permeability factor, when injected intracutaneously into guinea-pigs causes vasodilatation and increased permeability. It was of interest to test whether or not this permeability factor interacted with other plasma proteins to form plasma kinins which could account for part of this activity. Samples of the fractions G2 and G2α kindly supplied by Dr D. L. Wilhelm were tested for the formation of plasma kinins by incubation with pseudo-globulin solution. Neither of these fractions caused any contraction when added to the isolated guinea-pig ileum alone, but after 15 min incubation at 34° C with pseudo-globulin solution the

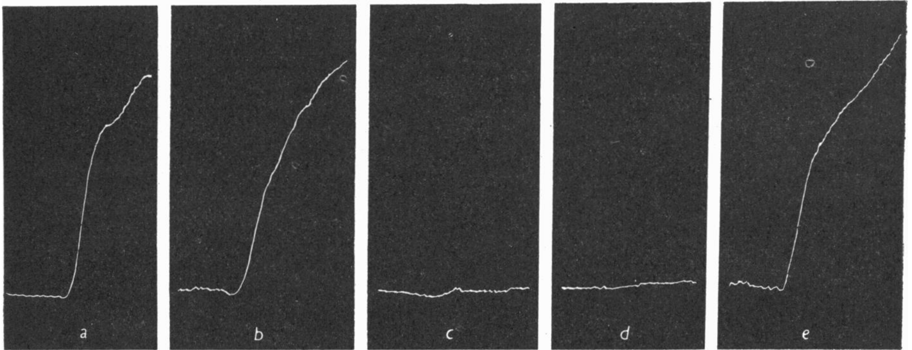

Fig. 10. Guinea-pig's ileum suspended in 15 ml. Tyrode solution. Permeability factor G2 0·3 ml. incubated with 0·1 ml. pseudo-globulin solution for 15 min, alone at (*a*), with antiplasmin 3 mg at (*b*) and with soya bean trypsin inhibitor 200 μg at (*c*). Permeability factor G2α 0·5 ml. incubated with 0·1 ml. pseudo-globulin solution for 15 min at (*d*). Permeability factor G2 0·3 ml. incubated for 15 min with 0·5 ml. G2α and 0·1 ml. pseudo-globulin at (*e*).

G2 fraction gave rise to smooth muscle stimulating activity while the purified G2α developed no such activity under the same conditions. This is illustrated in Fig. 10, which also shows that the development of smooth muscle stimulating activity by G2 is inhibited by soya bean trypsin inhibitor but not by antiplasmin. The failure of G2α to form smooth muscle stimulating activity on incubation with pseudo-globulin cannot be explained by the presence of an inhibitor in the G2α fraction which would prevent the development of this activity. This is illustrated in Fig. 10 (at *e*) which shows that after incubation with pseudo-globulin G2 still forms smooth muscle stimulating substance in the presence of G2α.

DISCUSSION

The present experiments show that plasmin, a proteolytic enzyme in blood, when incubated with plasma globulins, produces a plasma kinin which has vasodilator properties and stimulates isolated smooth muscle, but that the

precursor of plasmin, plasminogen, has neither proteolytic nor plasma kinin-forming activity, although these activities appear when plasminogen is activated to plasmin by the specific activator, streptokinase. The finding that the inactive precursor of plasmin forms a plasma kinin only when activated by its specific activator precludes the possibility that the plasma kinin-forming activity is due to an impurity in the plasmin preparations. An impurity which could have been responsible for this activity is kallikrein and the question naturally arises, what is the relationship between plasmin and kallikrein?

Kallikrein has been described by Frey *et al.* (1950) as a depressor substance which is capable of forming kallidin (a plasma kinin which is probably identical with bradykinin) when it is incubated with plasma proteins. Although it is not certain whether all the actions of kallikrein are due to the formation of plasma kinins or whether it has a vasodilator action of its own, it is clear that kallikrein is different from plasmin. Kallikrein is thermolabile and readily destroyed by acid, whereas plasmin retains most of its activity even after boiling at pH 2 for 30 min, which is, in fact, one step in the preparation of 'Mullertz' plasmin.

There is a close analogy between blood kallikrein and plasmin. Just as kallikrein is present in blood in an inactive form so plasmin is present as its inactive precursor, plasminogen. It has been shown that when plasma is treated with chloroform there is an increase in the proteolytic activity of the plasma which has been attributed to plasmin (Christensen, 1946). In the present experiments it has been shown that when plasma is treated with chloroform there is an appearance of smooth muscle stimulating activity (like that of a plasma kinin) and the development of this activity is inhibited by the presence of excess antiplasmin. Another interesting phenomenon which appears to involve the activation of plasmin is the increase in proteolytic activity in plasma which occurs on contact with glass. Armstrong *et al.* (1957) have observed that when freshly collected plasma comes into contact with glass it develops smooth muscle stimulating activity like that of a plasma kinin. This has been confirmed in the present experiments and it has also been shown that the development of this activity is inhibited not only by soya bean trypsin inhibitor but also by antiplasmin. Armstrong *et al.* have shown that not only smooth muscle stimulating and vasodilator plasma kinins are produced under these conditions but also that pain-producing plasma kinins are formed as well. It is therefore interesting to consider the possibility that plasmin forms not only plasma kinins with these actions but perhaps other plasma kinins exerting the diverse effects which form part of the tissue reaction to injury. For instance, it is possible to envisage the formation of plasma kinins with chemotaxic activity (like the leucotaxin of Menkin, 1938) under such conditions.

Frey, Kraut & Werle found enzyme-like depressor substances in other body fluids and tissues in addition to kallikrein in blood. These were also 'dubbed' kallikrein although there is some evidence that they are different from the kallikrein of blood. One site in which such an enzymic material was found is the salivary glands and recent experiments on functional hyperaemia in the salivary gland (Hilton & Lewis, 1955, 1956) have shown that functional hyperaemia in the submandibular salivary gland is the result of vasodilator plasma kinins (probably bradykinin) formed in the interstitial fluid as a result of the escape from the activated gland cells of this enzyme.

It was observed that in order for the maximum amount of plasma kinin to be formed it was necessary to incubate the plasmin and the pseudo-globulin for about 20 min. This delayed formation is quite different from the rapid formation of plasma kinin in the salivary gland. When saliva and pseudo-globulin are incubated the maximum plasma kinin formation occurs in 1–3 min (Hilton & Lewis, 1955). There may be a functional significance in these differences in the time of development of the vasodilator activity in the two cases. Hilton and I have shown that plasma kinin formation plays an important role in the functional vasodilatation in the salivary glands. In this case it would be essential that the vasodilator material be produced rapidly in order to account for the pronounced vasodilatation which occurs when the glands are activated. However, it is more likely that kinin formation by plasmin is important not in physiological mechanisms but in pathological conditions. For instance, in cases of tissue injury it is known that substances such as histamine are released locally from the injured tissue. These substances would increase the capillary permeability, allowing the plasminogen from the general circulation to enter the interstitial fluid and to make contact with the plasminogen activators (Astrup & Permin, 1947) escaping from the damaged cells. The activated plasminogen would then proceed to form vasodilator plasma kinins like bradykinin and possibly plasma kinins having other actions. Thus the secondary reactions of tissue injury which are known to be delayed in onset would be accounted for in this way.

A second instance where activation of plasminogen may play a role in pathological conditions is in generalized vascular shock, as suggested by Beraldo (1950). Under these conditions it might be envisaged that surface effects brought about by protein interaction, possibly acting in the same way as Armstrong *et al.* (1957) have shown a glass surface to act, would lead to an activation of plasminogen, not in the interstitial fluid as in the case of a localized reaction, but in the circulating blood, so that the plasma kinins formed in the circulation might play a role in this characteristic delayed general vascular collapse.

Another condition not yet discussed under which smooth muscle stimulating activity can develop in plasma is by dilution. The finding in the present experi-

ments that antiplasmin does not prevent the development of smooth muscle stimulating activity in plasma when it is diluted suggests that the activation by dilution is not due to activation of plasmin. It might well be that on dilution the kallikrein-inactivator complex is split as suggested by Schachter (1956) leaving kallikrein free to form plasma kinins.

Finally, the present experiments with the permeability factor of Miles & Wilhelm (1955) confirm their view that the purified factor does not act by forming plasma kinins, when it comes into contact with plasma proteins. However, it is possible that some of the effects exerted by the crude fraction G2 may be due to the presence of a plasma kinin-forming enzyme which does not behave like plasmin, as its action is not inhibited by antiplasmin but only by soya bean trypsin inhibitor.

SUMMARY

1. When plasmin, a proteolytic enzyme in blood, is incubated with plasma globulins, a plasma kinin is produced. The name plasma kinin includes all bradykinin-like polypeptides derived from plasma proteins.

2. A powder has been prepared from an incubated mixture of human plasmin and dog pseudo-globulin, containing a plasma kinin which on comparison with bradykinin was found to be indistinguishable from it.

3. Although crystalline trypsin was much more active than plasmin both as a proteolytic enzyme and as a plasma kinin-forming enzyme it was estimated that a plasmin preparation with a proteolytic activity equal to that of crystalline trypsin would be considerably more active as a plasma kinin-forming enzyme.

4. Plasmin differs from kallikrein, another enzyme in blood, in being thermostable and resistant to acid.

5. Plasminogen, the inactive precursor of plasmin, possesses neither proteolytic nor plasma kinin-forming activity, but acquires these properties on addition of the specific activator, streptokinase.

6. The development of smooth muscle stimulating activity when plasma is treated with chloroform or brought into contact with a glass surface is probably due to activation of plasminogen and the subsequent formation of a plasma kinin.

7. Activation of plasminogen does not account for the appearance of smooth muscle stimulating activity when plasma is diluted with saline, since the development of the activity is unaffected by antiplasmin.

8. The purified preparations of the permeability factor, G2α, do not form plasma kinins on incubation with plasma proteins, although the crude fraction G2 does contain a plasma kinin-forming enzyme.

9. It is suggested that plasmin plays a role in local tissue reactions when plasminogen is activated in the interstitial fluid with subsequent delayed formation of plasma kinins.

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