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THE EFFECT OF BRADYKININ, SERUM KALLIKREIN AND OTHER ENDOGENOUS SUBSTANCES ON CAPILLARY PERMEABILITY IN THE GUINEA-PIG

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Experiments with crude preparations of bradykinin have shown that they increase capillary permeability in the guinea-pig, rabbit (Holdstock, Mathias & Schachter, 1957) and man (Herxheimer & Schachter, 1959). The present experiments with highly purified preparations demonstrated that bradykinin is indeed a very potent enhancer of permeability. It was of interest therefore to study also the effect of serum kallikrein on capillary permeability, since this substance occurs normally in an inactive form in the blood of man and animals, and when activated releases kallidin, a polypeptide closely resembling bradykinin (see Frey, Kraut & Werle, 1950; Werle, 1955, 1960). Serum kallikrein was also found to be effective in increasing capillary permeability, and our results suggest that this activity is, in fact, due to the release of kallidin. The effects of serum kallikrein and bradykinin were similar qualitatively and quantitatively and both differed in a number of ways from the effect of histamine.

Since plasmin, the fibrinolytic enzyme in plasma, resembles kallikrein in that it exists in blood in an inactive but activable form (Müllertz, 1956; Biggs & MacFarlane, 1957), and has been found by some workers to be a potent releaser of a substance indistinguishable from bradykinin (Beraldo, 1950; Lewis, 1958), we compared its properties with those of serum kallikrein. Our experiments showed that kallikrein differed from plasmin in several ways. Plasmin was relatively ineffective in increasing capillary permeability and failed to release a smooth-muscle stimulating agent from plasma under conditions in which serum kallikrein was very active; on the other hand, plasmin was far more effective than kallikrein in producing fibrinolysis.

A preliminary communication of some of these results has previously been made to the Physiological Society (Bhoola & Schachter, 1959).

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METHODS

Capillary permeability. Albino guinea-pigs weighing 350–450 g were clipped of their fur and the skin of both flanks was depilated, beginning close to the dorsal mid line and extending 4–6 cm ventrally, between hips and shoulders. The guinea-pigs were injected intravenously with a 5 % solution of pontamine sky blue 6BX (E. Gurr) in saline via the saphenous vein (Miles & Miles, 1952) in a dose of 1·2 ml./kg. Intradermal injections of the various test substances in saline were then made in 0·1 ml. volumes with syringes of 0·25 ml. capacity fitted with fine, short-bevelled, intradermal needles (½ in. (1·3 cm), gauge No. 26). Needles were carefully sharpened on a fine stone before each experiment. The diameter of the blue lesion at the site of injection, due to dye leaving the circulation, was measured 25 min after the intradermal injection. The size of the lesion was taken as the mean of the largest diameter and the one at right angles to it, measurements being made on the unanaesthetized animal. Lesions measuring less than 3 mm are not significant since a mean diameter of this size represents 'traumatic' blueing due to the injury of injection.

Bradykinin, serum kallikrein and histamine were injected in concentrations of 200, 50, 10, 2 and 0·5 μg/ml. in 0·1 ml. volumes, each substance in a row on the flank of the guinea-pig, as is shown in Pl. 1, fig. 1. In order to derive dose–response curves for each substance it was found necessary to vary only the positions of the rows of different substances relative to one another on the flank. Both flanks of the animal were injected in succession in this way, the lesion diameters measured, and dose–response curves obtained, each point on the curve representing the mean diameter of a minimum number of six lesions in at least three animals. This procedure was adopted because of its relative simplicity and because we found in preliminary experiments that complete randomization of different doses of these substances in Latin square distributions on the flank revealed no significant variation in the size of lesions produced by the same concentration of a substance in different locations. This is correct, provided that the region of the flank injected is confined between hip and shoulder, and does not extend ventrally for more than 4 cm from the dorsal mid line. Injections made beyond this limit ventrally produce progressively larger lesions with the same concentration of drug.

To test the effect of the antihistamine drug mepyramine the following procedure was adopted. The various test substances were injected intradermally after the animal had been injected with pontamine blue, and measurements of the lesions were made 25 min later as already described. The animal was then injected with mepyramine (0·1 mg/kg) via the saphenous vein, and intradermal injections of the test substances were repeated on the other flank. Twenty-five minutes later these lesions were measured and compared with those obtained in the same animal before treatment with mepyramine.

Soya-bean trypsin inhibitor (SBTI) and ovomucoid trypsin inhibitor (OI) were incubated for 30 min at room temperature with bradykinin, kallikrein or histamine when tested for their inhibitory activity. In some animals a concentration of SBTI of 200 μg/ml. itself produced an increase in permeability. Animals used for testing were therefore given an initial injection of SBTI to ascertain that they did not react to the drug in the concentration being used.

Fibrinolysis. For measuring fibrinolysis the fibrin plate method described by Astrup & Müllertz (1952) was used, but with some minor modifications which we found necessary to provide a suitable consistency of fibrin clot for quantitative measurements of lysis under our conditions. The fibrin clots were made on Petri dishes of 10 cm diameter by clotting 8 ml. of 0·15 % (clottable protein) of purified human fibrinogen solution with 0·17 ml. of bovine thrombin (17 u.). The fibrin plates were incubated for 1 hr at 37° C, a procedure which provided a clot of suitable and constant consistency. A drop of 0·03 ml. of each enzyme solution in veronal buffer was then carefully placed on the fibrin surface from a micropipette. The plate was divided into four compartments and at least eight spots could

be applied to each plate. They were incubated for approximately 16 hr at 37° C and the diameters of each lysed area measured. Inhibitors (SBTI and OI) were made up in the same buffer and mixed with the test solutions for 30 min before applying a drop of the mixture to the fibrin plate in the same way.

Isolated guinea-pig ileum. A loop of ileum was suspended in an 18 ml. bath of Mg-free Tyrode solution containing atropine and mepyramine (10^{-5} g/l.).

Drugs and other materials

Bradykinin was prepared by the action of crystalline trypsin on heated ox serum globulin (Holdstock *et al.* 1957) and purified chromatographically so that it contained 900 u./mg freeze-dried material, in terms of the units of Rocha e Silva, Beraldo & Rosenfeld (1949) when tested on the guinea-pig ileum. This bradykinin preparation was kindly provided by Mr W. A. Jones and Dr I. M. Lockhart.

Serum kallikrein was prepared by the casein adsorption method (Werle, 1936) and purified by fractionation with acetone, alcohol and ammonium sulphate. Our two preparations, kindly provided by Professor E. Werle, contained 3 and 35 kallikrein u./mg protein. The kallikrein solutions, containing a trace of toluol as a preservative, were dialysed against saline for several hours in the cold before experiments. Solutions were kept frozen until required and once thawed were not used again, as activity was found to decline with thawing and re-freezing of the purer preparations. The weights of serum kallikrein are expressed as weights of protein, based on nitrogen determinations.

Plasmin was a freeze-dried preparation made from human plasminogen activated by streptokinase. It was provided by Merck, Sharp and Dohme, Ltd.

Thrombin. Bovine thrombine, approximately 30 u./mg (S. Maw, Ltd.)

Fibrinogen. Purified human fibrinogen, 80–90% clottable protein (Blood Products Laboratory, Lister Institute, Elstree).

Salivary kallikrein was a freeze-dried preparation from human saliva, as described by Holdstock *et al.* (1957).

Trypsin. Crystalline trypsin (Armour), 2640 u./mg.

Serum and plasma. Human blood, for plasma, was obtained by venepuncture and collected in heparin. Guinea-pig serum or plasma (+ heparin) was obtained by cutting the jugular vein, after killing the animal by a blow on the head. Some of these were heated to 56° C for 3 hr.

Veronal buffer was made up according to the method of Astrup & Müllertz (1952) and consisted of 662 ml. 0·1 M sodium diethyl barbiturate mixed with 338 ml. 0·1 M-HCl diluted with 320 ml. distilled water. The pH of this buffer was 7·8 and ionic strength (I) 0·05.

Soya bean and ovomucoid trypsin inhibitors were crystalline preparations (Worthington).

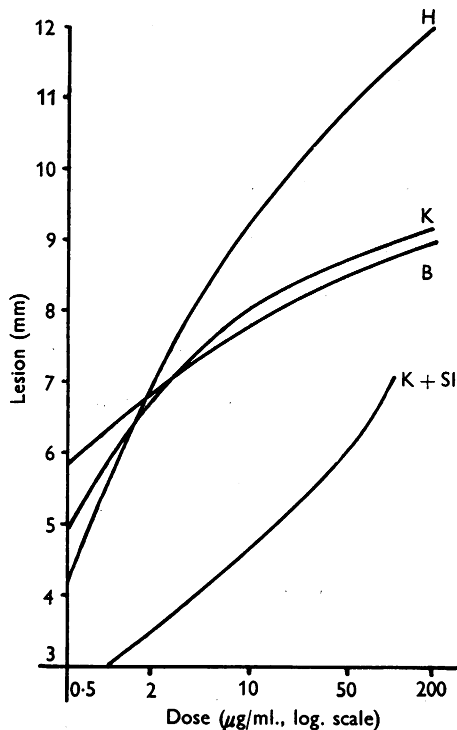
Histamine, atropine and mepyramine were used as acid phosphate, sulphate and maleate respectively; weights of histamine are expressed as base.

RESULTS

Capillary permeability

Comparison of bradykinin, serum kallikrein, histamine and plasmin. Bradykinin and serum kallikrein, when injected intradermally into guinea-pigs with circulating pontamine blue dye, regularly increased capillary permeability and produced lesions of a more intense blue colour than those produced by histamine. In general, increased blueing appeared within 2–3 min of intradermal injection of these substances, whereas the delay was 4–6 min after injection of histamine. Bradykinin and kallikrein,

though still impure, were more effective than histamine in the lower concentrations. The dose–response curves for bradykinin and kallikrein, however, unlike that for histamine, showed a pronounced flattening with the higher concentrations (Pl. 1, fig. 1; Text-fig. 1). The size of the lesion was fully developed in 20–30 min in all instances, and no significant increase was detected after 2 hr.



Text-fig. 1. Dose–response curves of histamine (H), serum kallikrein (K) and bradykinin (B) in increasing capillary permeability in guinea-pigs with circulating pontamine blue dye. 0·1 ml. of each solution was injected intradermally. K + SI, serum kallikrein + soya-bean trypsin inhibitor (200 μg/ml.) incubated at room temperature for 30 min before injection of 0·1 ml. of mixture.

A preparation of human plasmin, which had high fibrinolytic activity, was without effect on capillary permeability. In three animals tested a significant response to intradermal injections began to appear only at concentrations of 500 μg/ml., indicating activity approximately 1000 times less than that of the other substances (Pl. 1, fig. 2).

Effects of inhibitors. Soya bean trypsin inhibitor (SBTI) in a concentration of 200 μg/ml. reduced the permeability-enhancing property of serum kallikrein 50–100 times (Text-fig. 1) on incubation (30 min; room temperature) with solutions of kallikrein containing 0·25–3·0 u./ml., or

less. The effects of higher concentrations of SBTI could not be studied, because in some guinea-pigs a concentration of 200 μg/ml. of SBTI itself produced a significant increase in capillary permeability. Ovomucoid trypsin inhibitor (OI), in concentrations up to 500 μg/ml., failed to affect the permeability-increasing activity of kallikrein under the same conditions. Pre-treatment of the animal with mepyramine (0·1 mg/kg, I.V.) was also without effect (Pl. 1, fig. 3).

The action of bradykinin was unaffected by incubation with SBTI or OI as described above. Also, the dose–response curve of animals pre-treated with mepyramine remained unchanged.

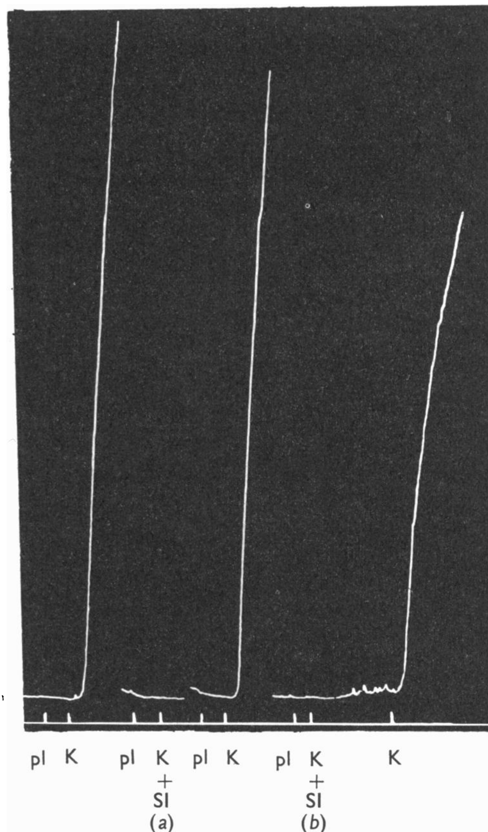
Histamine, like bradykinin, was unaffected by SBTI or OI throughout its activity range, but was practically completely antagonized by pre-treatment of the animal with mepyramine (Pl. 1, fig. 3).

Release of kallidin by serum kallikrein and by plasmin

Serum kallikrein was a potent releaser of kallidin not only from the plasma of the guinea-pig, but also from those of man, ox, dog, cat and rabbit. Heated (56° C, 3 hr) dialysed plasma was used in most experiments (to eliminate the release of kallidin by dilution, and to destroy the inactivators of kallidin and of kallikrein), but similar results were obtained with unheated plasma. We could readily detect the release of kallidin from human or guinea-pig plasma with less than 0·15 u. of kallikrein, using the isolated guinea-pig ileum as a test object. This was observed regularly either when plasma (0·2–0·5 ml.) was incubated with kallikrein for 1 min and then tested, or when 0·5 ml. plasma was added to the bath containing the ileum and the kallikrein then added to the bath contents. In this case the final concentration of kallikrein was approximately 0·01 kallikrein u./ml. or 0·3 μg protein/ml. of our purer preparation. In terms of the purest preparations described by the German workers (Frey *et al.* 1950), this represents a concentration of less than 0·01 μg kallikrein/ml. The release of kallidin by serum kallikrein is readily prevented by incubation of the latter with SBTI. Text-figure 2 shows the kallidin-releasing action of kallikrein on heated guinea-pig plasma and the inhibition of this release by preliminary incubation of the kallikrein solution wilh SBTI. This figure also shows that after inhibition of release by SBTI, kallidin may still be released from a mixture of plasma and inactivated kallikrein by addition of excess kallikrein.

A comparison of a potent fibrinolytic preparation of human plasmin (see below) with serum kallikrein indicated that the latter was at least several hundred times the more active of the two in releasing kallidin from heated (56° C, 3 hr) guinea-pig or human plasma. In fact, even when 1 mg plasmin was added to the test bath containing heated plasma, evidence of

release of a smooth-muscle stimulant was doubtful. Similar results were obtained with unheated plasma. The rapid release of kallidin from guinea-pig and from human plasma under conditions when equal or greater amounts of plasmin were ineffective is shown in Text-fig. 3. This figure

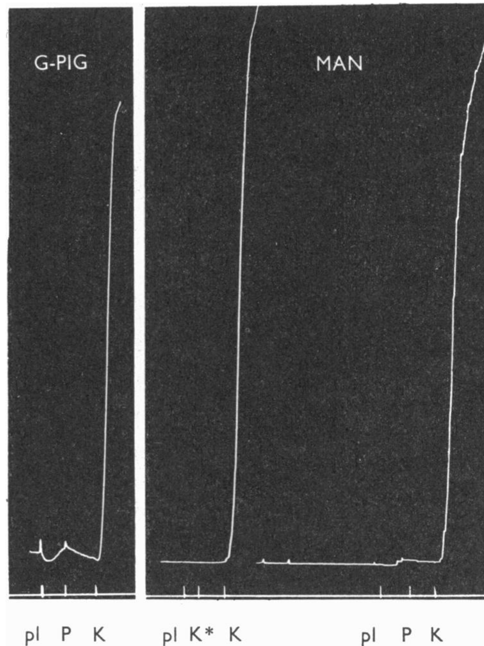


Text-fig. 2. The release of kallidin by serum kallikrein and inhibition of release by soya-bean trypsin inhibitor (SI). Contractions of guinea-pig ileum in 17 ml. bath of Mg-free Tyrode solution (34° C) containing atropine and mepyramine (10^{-5} g/l.). Continuous line indicates bath not washed out. pl, 0·5 ml. dialysed, heated (56° C, 3 hr) guinea-pig plasma; K, serum kallikrein (0·5 u. 200 μg); K + SI, serum kallikrein (0·5 u.) incubated with soya-bean trypsin inhibitor (200 μg/ml., (*a*); 20 μg/ml., (*b*)) for 10 min at 35° C before addition to bath.

also shows that kallikrein, if the pH of the solution is first lowered with hydrochloric acid to pH 2 for 20 min, is completely inactivated. Plasmin, however, is reported to be very stable at this pH (Müllertz, 1956). Human salivary kallikrein and crystalline trypsin preparations were also very effective in releasing a smooth-muscle stimulant from heated guinea-pig or human plasma under these conditions.

Fibrinolytic activity

The fibrinolytic activity of plasmin, trypsin, serum kallikrein and salivary kallikrein was measured on fibrin plates. Plasmin was the most effective of these on a weight basis, regularly producing detectable lysis when 0·03 ml. of a solution containing 0·75 μg/ml. was placed on a fibrin plate. Such a solution of plasmin produced a lysed ring of fibrin with a mean diameter of 6·6 mm. With 0·03 ml. volumes, containing plasmin in concentration of 10

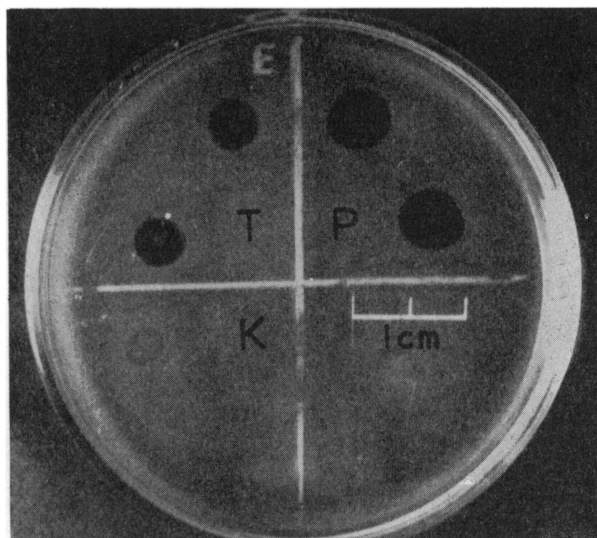


Text-fig. 3. Contractions of isolated guinea-pig ileum in Tyrode solution containing atropine and mepyramine. Continuous line indicates bath not washed out. The release of kallidin from guinea-pig (left) and human plasma (right). pl, dialysed heated plasma (0·5 ml. g-pig; 1·0 ml. man) P, human plasmin (1·0 mg); K, serum kallikrein (0·25 u.); K*, serum kallikrein (0·25 u. + HCl) at pH 2 for 20 min before neutralization and addition to bath.

and 20 μg/ml., the mean diameters of the lysed areas were approximately 10·9 and 15·0 mm, respectively. Trypsin was also detectable in solutions containing 0·75 μg/ml., but the lysed area was smaller than with plasmin. The minimal concentration of trypsin detectable was approximately five times greater than that of plasmin. Serum kallikrein showed fibrinolytic activity in high concentrations, but activity was not demonstrated until concentrations of 15–50 μg/ml. were employed. The activity at this concentration was less than that of solutions containing 0·75 μg/ml. trypsin

or plasmin. The relative activities of plasmin, trypsin, and kallikrein in lysing human fibrin are shown in Text-fig. 4.

The fibrinolytic activity of trypsin and plasmin were both sensitive to SBTI, effective concentrations of each of these enzymes being completely inactivated by the same or even lower concentrations of SBTI. Both enzymes showed a much less sensitivity to OI, although the fibrinolytic activity of trypsin was reduced by high concentrations of this inhibitor.



Text-fig. 4. Lysis of human fibrin (16 hr, 37° C); 0·03 ml. of each solution in concentration of 10 μg/ml. P, human plasmin; T, crystalline trypsin; K, serum kallikrein.

DISCUSSION

Our highly purified preparations of bradykinin, though not yet pure, were more effective than histamine on a weight basis in increasing capillary permeability in the lower concentrations. It is difficult, however, to compare the relative potency of histamine and of bradykinin in increasing permeability because of the different shapes of the dose–response curves, and because of the apparent greater accumulation of dye in the lesions produced by bradykinin. Since pure bradykinin has been estimated to contain 5000–10,000 u./mg, it would be 5–10 times still more potent than our preparation. Bradykinin preparations, which varied from 7 to 1000 u./mg (assayed on the guinea-pig ileum) showed parallel gut-stimulating and permeability-enhancing properties, indicating that these properties reside in the same molecule. The recently prepared pure bradykinin has, in fact,

been found to retain the permeability-increasing activity (D. F. Elliott, E. W. Horton & G. P. Lewis, personal communication).

The remarkable activity of serum kallikrein in increasing permeability is of interest in so far as high concentrations of this substance exist in blood in an inactive but activable form (Frey *et al.* 1950). The close similarity of its dose–response curve to that of bradykinin (Text-fig. 1) suggests that this effect is due to the release of the polypeptide, kallidin, which kallikrein releases from a precursor in plasma and lymph (see Werle, 1960; Schachter, 1960). This view is supported by the fact that our most active serum kallikrein preparation was more active in releasing kallidin than trypsin was in releasing bradykinin, and that this property, like the permeability-increasing property, was greatly reduced by incubation with SBTI but not with OI.

The fact that serum kallikrein is probably an enzyme which is present in an inactive but activable form in plasma raises the question of its possible relationship to plasmin, the fibrinolytic enzyme (or enzymes) in plasma (Müllertz, 1956; Biggs & MacFarlane, 1957). This is particularly relevant since it has been stated that human plasmin is a very potent releaser of plasma kinin (Lewis, 1958) although this release appears to be rather slow. Our experiments indicate that there is no correlation between fibrinolytic activity and the rapid release of a smooth-muscle stimulant from plasma by serum kallikrein, plasmin, trypsin and salivary kallikrein. In fact, plasmin, which had the greatest fibrinolytic activity, was ineffective in releasing a smooth-muscle stimulant from plasma in concentrations approximately 500 times greater than that in which our best serum kallikrein preparation released kallidin within a few seconds of its addition to plasma. These facts indicate that serum kallikrein has different substrate requirements from that of the highly active fibrinolytic enzyme of plasma. It is of interest in this connexion that human salivary kallikrein, a potent releaser of kallidin, completely failed to lyse human fibrin, even in high concentrations. Further chemical studies are desirable to clarify the interesting problem of the substrate specificities of the different kallikreins.

The prominent pharmacological properties of serum kallikrein (and of the other kallikreins in so far as they have been studied) are the vasodilator, permeability-enhancing, and kallidin-releasing actions. It seems unlikely that these properties will dissociate with purification (Habermann, 1959). The suggestion (Schachter, 1956; Herxheimer & Schachter, 1959) that the release of kallidin-like substances from plasma by dilution, or by contact with glass, is due to the activity of kallikrein, is strengthened by our demonstration of the potent kallidin-releasing action of serum kallikrein. Similarly, the permeability-enhancing activity of diluted plasma (Mackay, Miles, Schachter & Wilhelm, 1953; Miles & Wilhelm,

1955) and of the permeability globulins in fractionated plasma (Wilhelm, Miles & Mackay, 1955; Mill, Elder, Miles & Wilhelm, 1958), or in inflammatory exudates (Spector, 1957), may all be due to serum kallikrein. For the same reasons it is possible that the 'enzyme' activated in plasma by glass contact (Armstrong, Jepson, Keele & Stewart, 1957), which causes the release of plasma kinin and accelerates clot formation (Margolis, 1958), is also serum kallikrein. Also, those individuals whose blood shows a prolonged clotting time *in vitro*, and who show no haemorrhagic disturbance, but are deficient in the so-called Hageman factor in their plasma (Ratnoff & Colopy, 1955) may, in fact, be deficient in the inactive kallikrein precursor, since their plasmas do not release plasma kinin on contact with glass or kaolin (Margolis, 1958). It would be of interest, therefore, to measure the amount of active kallikrein extractable from their blood by the usual procedures (Frey *et al.* 1950). If serum kallikrein is in fact the active agent involved in all these observations, a possibility which seems not unlikely, it would provide a simplified explanation of many diverse and apparently unrelated observations. More experiments are required to establish definitely whether or not this is the correct explanation of these facts.

SUMMARY

1. Bradykinin, when injected intradermally, is one of the most effective enhancers of capillary permeability, as measured by its ability to increase permeability to a circulating dye in the guinea-pig. The effect differs from that of histamine in that there is a greater accumulation of dye in the lesions, and that there is an earlier pronounced flattening of the dose–response curve in respect to lesion size. Its action is unaffected by treatment of the animal with mepyramine or by incubation of the bradykinin solution with soya bean or ovomucoid trypsin inhibitors.

2. Serum kallikrein is also very effective in increasing capillary permeability in the guinea-pig. Its effects resemble those of bradykinin both in the appearance of the lesion and in the shape of the dose–response curve. This action of kallikrein is unaffected by treatment of the animal with mepyramine but is reduced if the solutions are incubated with soya-bean trypsin inhibitor before injection. Ovomucoid inhibitor is without effect. These results suggest that this action of kallikrein is mediated by the release of kallidin from plasma. Serum kallikrein is a very potent releaser of kallidin from heated (56° C, 3 hr) or unheated plasma or serum. This action is readily suppressed by incubation with soya-bean trypsin inhibitor but not by ovomucoid inhibitor.

3. Plasmin is practically without effect on capillary permeability, and fails to release a smooth-muscle stimulant from plasma under conditions

in which trypsin, salivary kallikrein and serum kallikrein are very effective. There is no relationship between the ability of these substances to produce lysis of fibrin and to release kallidin or bradykinin.

4. The possible activation of serum kallikrein in a number of procedures (glass contact, dilution, etc.) which result in the appearance of smooth-muscle-stimulating or permeability-enhancing activity in plasma is discussed. Also, the possible relationship of various other substances in plasma (permeability globulins, contact factor, Hageman factor) to serum kallikrein is considered.

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EXPLANATION OF PLATE

Fig. 1. Skin of guinea-pig with circulating pontamine blue dye showing lesions produced by intradermal injections (0·1 ml.) of B, bradykinin; H, histamine; K, serum kallikrein.

Fig. 2. Skin of guinea-pig with circulating pontamine blue dye. Lesions produced by intradermal injections (0·1 ml.) of H, histamine; P, human plasmin.

Fig. 3. Skin of both flanks of same guinea-pig with circulating pontamine blue dye. Right: skin of one flank with intradermal injections of approximately equal activities of bradykinin (B), histamine (H), and serum kallikrein (K). Left: skin of other flank similarly injected after mepyramine (0·1 mg/kg) had been administered intravenously; only the histamine response is suppressed.

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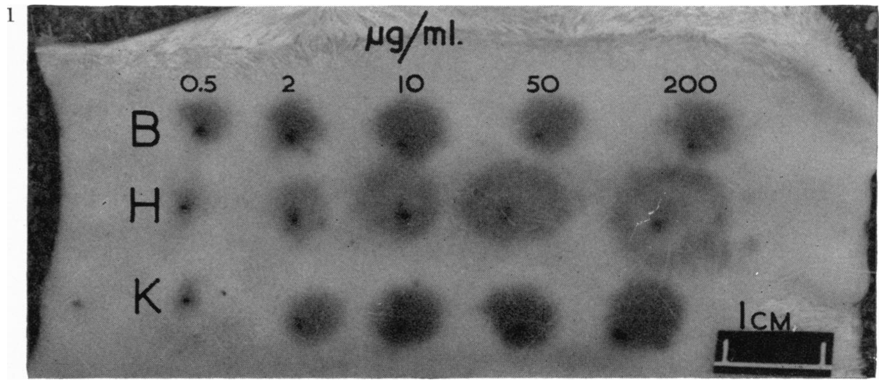

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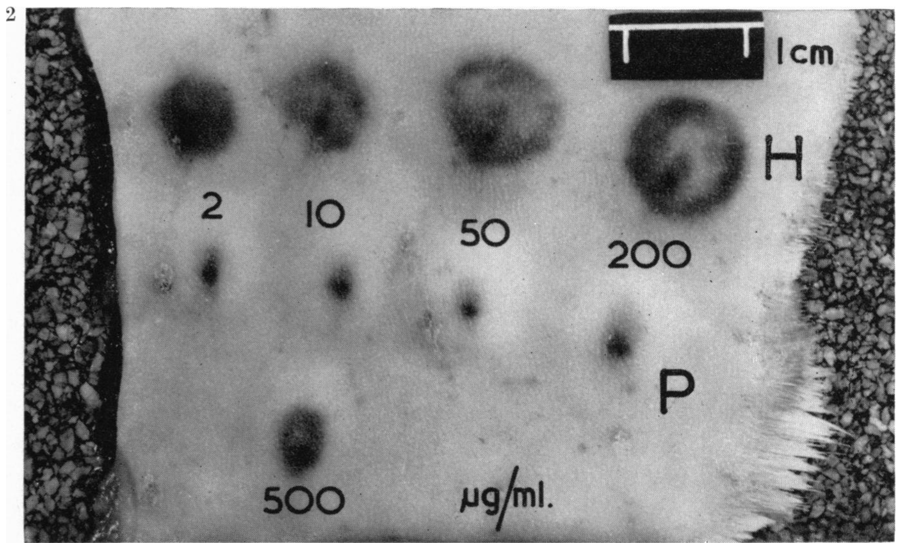

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