J. Physiol. (1963), **166**, *pp.* 496–513
With 6 text-figures
Printed in Great Britain

OBSERVATIONS ON INTRINSIC KININ-FORMING FACTORS IN HUMAN PLASMA: THE EFFECT OF ACID, ACETONE, CHLOROFORM, HEAT AND EUGLOBULIN SEPARATION ON KININ FORMATION

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(*Received* 14 *March* 1962)

Plasma kinins, i.e. polypeptides derived from plasma proteins, which dilate blood vessels and contract or relax smooth muscle (Lewis, 1958, 1960), can be formed by a number of enzymes, some of which occur in plasma itself. In human plasma several kinin-forming factors have been described and named independently by various groups of workers. Thus, the *fibrinolytic* system in plasma has been held responsible for several modes of kinin formation (Beraldo, 1950; Lewis, 1958; Hamberg, 1959). *Hageman Factor* (HF), which initiates the clotting response of blood to silica and other activating surfaces, also leads to kinin formation. In Hageman trait the patients lack HF and both the clotting and the kinin-forming reactions of blood to foreign surfaces are deficient. According to Margolis (1959), contact-activated and adsorbed HF first converts a postulated enzymic precursor, 'component A', into 'activated A' (cf. Table 1). The latter remains in solution in the plasma, where it interacts with 'component B' to form plasma kinin. The plasma *kallikreins* may achieve all their effects by releasing a plasma kinin, kallidin (Frey, Kraut & Werle, 1950, p. 179). A *permeability factor* (PF; Miles & Wilhelm, 1960) may also form plasma kinin (Armstrong, Jepson, Keele & Stewart, 1957). In some instances plasma kinin formation induced by the same agent has been attributed to different intrinsic factors. Thus, Horton (1959) attributed kinin formation in acid-treated dog plasma to a surviving remnant of kallikrein. Hamberg (1959) and Rocha e Silva (1960) thought that in rat, guinea-pig, rabbit and human plasma heated at pH 2 kinin was formed by plasmin. In spite of this lack of co-ordination between the work of different schools, it is generally agreed that the various modes of intrinsic plasma kinin formation are determined by the following basic mechanisms in plasma: (i) a forming system, probably of enzymic nature; (ii) a protein substrate ('kininogen'), from which kinin is released; (iii) inhibitors and/or inactivators of the forming system; (iv) inactivators of the formed kinin ('kininase').

In the present experiments some chemical and functional characteristics of intrinsic kinin formation in human plasma were studied. It was hoped that the information obtained in this way might help to clarify the relationship between the various described agents. The work described in this and the following paper has in part been reported in preliminary communications (Eisen & Keele, 1960*a*, *b*).

METHODS

Ballotini. Graded glass micro-spherules, 0·1 mm in diameter (English Glass Co., Leicester), with a surface area of the order of 200 cm^2/g, were used. They were measured by their dry volume, on the basis that 1 ml. of ballotini weighs 1·7 g.

Kaolin. (Hydrated aluminium silicate, British Drug Houses.) Its surface area is given as 10 m^2/g.

Barium sulphate for adsorption of plasma was washed as prescribed by Biggs & Macfarlane (1957, p. 388).

Michaelis's veronal acetate-saline buffer was prepared according to the method of Biggs & Macfarlane (1957, p. 387).

Sodium phosphate buffers were prepared from 0·1 M stock solutions of NaH_2PO_4 and Na_2HPO_4.

Varidase (Lederle). Bottles containing 100,000 u. streptokinase and 25,000 u. streptodornase.

Human serum kallikrein, 0·35 Frey units/mg, was prepared and kindly given by Dr M. E. Webster of the National Institute of Health, Bethesda, Maryland, U.S.A.

Hog serum kallikrein, 25 Frey units/ml., was prepared by Professor E. Werle, and obtained through the courtesy of Dr M. Schachter of University College London.

Soya bean trypsin inhibitor (SBTI), *Lima bean trypsin inhibitor* (LBTI) and *Ovomucoid trypsin inhibitor* (OTI) (Nutritional Biochemicals Corporation) were used dissolved in NaCl solution, 0·9 g/100 ml., or in buffer solution.

Collection of blood. Human blood was collected by venepuncture, silicone-coated syringes and needles being used, and transferred into polythene centrifuge tubes.

Anticoagulants were used as follows: heparin 1–5 u./ml., sodium oxalate 13·4 mg/ml., trisodium citrate 4 mg/ml. blood.

Plasma which has not been in contact with glass is called 'pre-active' or intact plasma (PAP; Armstrong *et al.* 1957). Activation of the kinin-forming system in such PAP with silica surfaces was performed as described by Margolis (1958). The plasma components A and B involved, according to the same author (1958, 1959), in the kinin-forming response to contact, were removed from plasma following his methods of measured extensive exposure to glass. To consume all the component B, plasma was rotated with 0·17 g ballotini/ml. for 10–15 min. Depletion of both B and A was achieved by exposure to 1 g ballotini/ml. for at least 2 hr. After separation from the glass, treated plasma was left at room temperature for at least 6–8 hr in polythene containers, to allow any generated kinin and 'forming activity' to decay. Renewed glass contact forms in B-depleted plasma (B-dep) soluble kinin-forming activity ('activated component A'), but no kinin; in (A + B)-depleted plasma ((A + B)-dep) glass still adsorbs HF, but produces neither activated A, nor kinin. In the present text, the terms PAP, B-dep and (A + B)-dep are used only in relation to the described treatments with glass and not in relation to any other plasma treatments.

'Coating' of ballotini with plasma constituents. Ballotini were immersed in plasma or its derivatives for 5 min, and then washed several times with NaCl solution 0·9 g/100 ml. Such ballotini are coated with HF and other plasma constituents.

Heating of plasma. Before heating samples were adsorbed with 100 mg $BaSO_4$/ml. for 10 min.

Methods for removal or destruction of fibrinolytic inhibitors

Acid treatment of plasma. The method of Alkjaersig, Fletcher & Sherry (1959) was used, except where modified as described. With this method the re-neutralized plasma is 5 times diluted.

Precipitation with M-*NaCl at* pH 2 was carried out according to the method of Troll & Sherry (1955).

Precipitation with cold acetone was performed according to the method of Lassen (1958).

Separation of plasma euglobulins. Two methods were used: (*a*) precipitation with dilute acetic acid (Milstone, 1941); (*b*) precipitation by dilution with distilled water and saturation with CO_2 (von Kaulla & Schultz, 1958).

Treatment with chloroform was performed according to the methods of Tagnon (1942) or Austen (1960).

Procedure

Isolated rat uterus. Virgin albino rats weighing 120–200 g were injected intramuscularly 16–18 hr before use with stilboestrol 20 μg/100 g body weight. One horn of the uterus was suspended in a 10 ml. bath at 30° C in de Jalon's uterus fluid. Isotonic contractions against a load of 1–1·25 g were recorded with a fivefold magnification. In many experiments an auxotonic system (Paton, 1957) was set up. A cylindrical float was suspended, from the writing limb of the lever, in water. In this way the basic load on the resting uterus was increased when the organ contracted. The increase was linear with contraction at any deflexion of the lever. Convenient slopes of the dose–response curve were obtained with load increases of 0·1–1·0 g/cm uterine contraction. Up to a twentyfold dose range could be covered whilst a reasonable discrimination between doses was preserved.

The isolated guinea-pig ileum and rat duodenum were prepared as described by Gaddum & Horton (1959).

Cantharidin blister base in man. The method developed by Keele and his collaborators was used (Armstrong, Dry, Keele & Markham, 1953; Armstrong *et al.* 1957).

The cat blood pressure was recorded from the common carotid artery. The animals were given pentobarbitone sodium (60 mg/kg intraperitoneal) and pentamethonium bromide (10 mg/kg) I.V.

Rat blood pressure. The method of Crawford & Outschoorn (1951) was followed with some modifications. The animals were given urethane (175 mg/100 g body weight) subcutaneously and pentamethonium bromide 1·2 mg/100 g I.V. An indwelling cannula in a tail vein was used for intravenous applications.

RESULTS

Formation of kinin in acid-treated plasma

When human plasma which had not been exposed to contact with glass ('pre-active plasma', PAP) was acid-treated by the method of Alkjaersig *et al.* (1959), stable kinin-like activity appeared spontaneously, that is without any further activating procedure. No difference was detected between this kinin and kinin formed by contact of plasma with glass, when tested on the isolated rat uterus, the guinea-pig ileum, the rat duodenum and the cantharidin blister base in man. Kinin developing in acid-treated plasma was rapidly destroyed by non-acidified plasma and by chymotrypsin. At room temperature kinin was formed slowly. Its concentration continued to increase for 4–6 hr, and then reached a level

which remained constant for several hours. The kinin which after exposure to pH 2 for 15 min eventually developed in 1 ml. of human PAP was in most cases equivalent to 1–10 μg of synthetic bradykinin (Fig. 1).

The prolonged formation and the stability of the resulting kinin suggested that acid treatment had destroyed the inactivators of the forming enzymes as well as those of kinin.

Nature of kinin-forming process. The time course of kinin development suggested an enzymic process. This impression was strengthened by the finding that in the range of 3–37° C the rate of formation increased with the temperature.

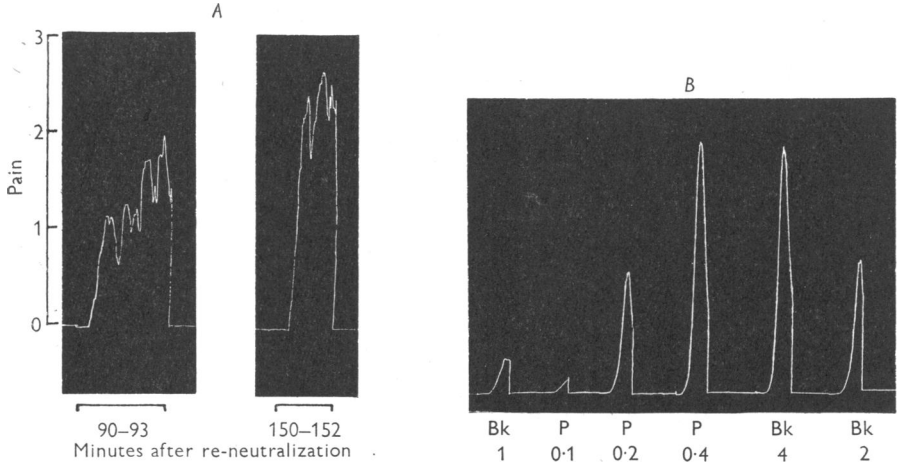


Fig. 1*A*. Pain-producing effect of acid-treated human plasma (PAP) applied to the blister base at 90–93 min and at 150–152 min after re-neutralization. Ordinate = intensity of pain: 1, slight; 2, moderate; 3, severe.

B. Rat uterus preparation: comparison of acid-treated PAP (P), 10 hr old, with synthetic bradykinin (Bk). The concentrations in the bath fluid are given for Bk in ng/ml., and for P in μl./ml. The kinin formed in 1 ml. of this PAP was equivalent to 10 μg of synthetic bradykinin.

The kinin formation after acid treatment was inhibited by soya bean trypsin inhibitor (SBTI) 0·2–1 mg/ml., and by lima bean trypsin inhibitor (LBTI) 4–10 mg/ml.; ovomucoid trypsin inhibitor (OTI) was not effective in concentrations of 20 mg/ml.

The slow, spontaneous, kinin formation in acid-treated PAP did not represent the maximum reaction rate of the forming factors present in this preparation. The rate of kinin formation in acid-treated plasma diluted 5 times or more could be greatly enhanced by incubating this preparation with higher concentrations of substrate. In the experiment of Fig. 2 this additional substrate was supplied by non-acidified undiluted PAP (*a–e*), or by heated PAP in which essential kinin-forming enzymes

had been destroyed (*f–i*). The kinin formation in acid-treated PAP was, of course, also accelerated when the concentration of active forming enzyme in the preparation was raised, e.g. by contact with HF-coated glass (test *c*).

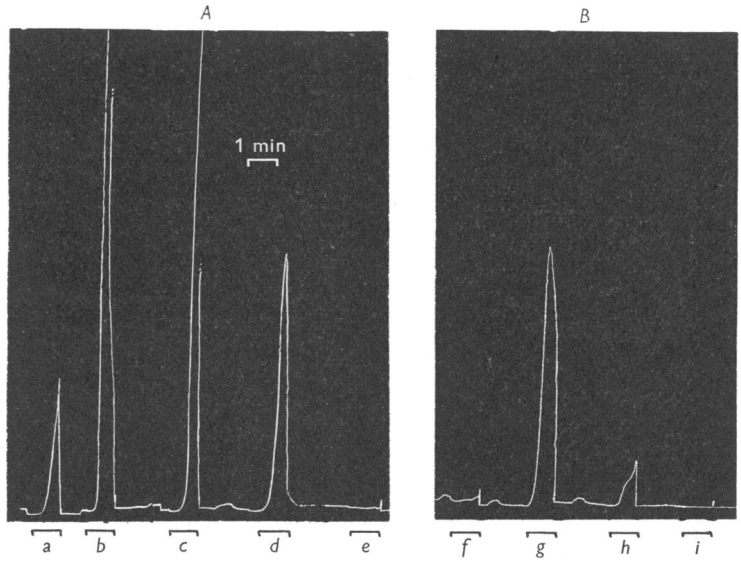
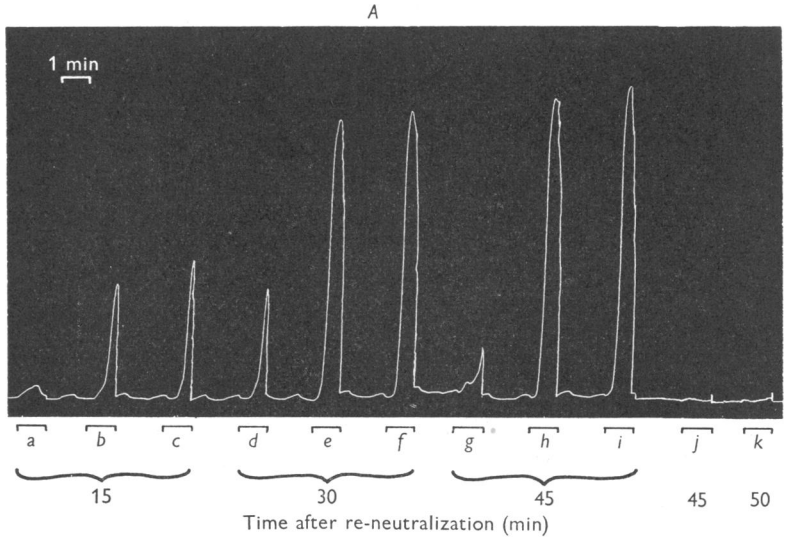


Fig. 2. Evidence that the rate of kinin formation in acid-treated PAP, 5 times diluted, is below the maximal reaction rate of the forming factors. Rat uterus. In *A* additional substrate was supplied as untreated PAP; in *B*, as PAP heated at 56° C for 3 hr to destroy kinin-forming enzymes. Tests at the following times after re-neutralization. *A* (*a*) = 0·05 ml. of acid-treated PAP at 10 min; (*b*) = 0·05 ml. of (*a*) + 0·05 ml. of PAP, incubated at 12–15 min and tested at 15 min; (*c*) = 0·05 ml. of (*a*) tested at 20 min, after 3 min rotation with HF-coated ballotini; (*d*) = 0·05 ml. of (*a*) at 25 min; (*e*) = 0·1 ml. of PAP. *B* (*f*) = 0·05 ml. of acid-treated PAP at 10 min; (*g*) = 0·05 ml. of (*f*) + 0·05 ml. of heated PAP, incubated at 12–15 min and tested at 15 min; (*h*) = 0·05 ml. of (*f*) at 20 min; (*i*) = 0·1 ml. of heated PAP.

Relationship of the kinin-forming factor in acid-treated plasma to plasma kallikrein. From the described experiments it was not possible to determine whether the kinin formation was due to an acid-resistant enzyme of the plasmin type, or to a remnant of an acid-labile enzyme, such as plasma kallikrein or permeability factor. To answer this question PAP was acidified for 15 min at room temperature with $\frac{1}{2}$, 1 and 2 vol. of N/6-HCl to pH 4·8, 2·3 and 1·2 respectively (Fig. 3*A*). After re-neutralization only small transient kinin formation occurred in the sample treated at pH 4·8. The sample exposed to pH 1·2 formed kinin at a slightly faster rate than the sample exposed to pH 2·3. Acidification with N-HCl to pH 0·5 inactivated the responsible enzyme. Samples of plasma which



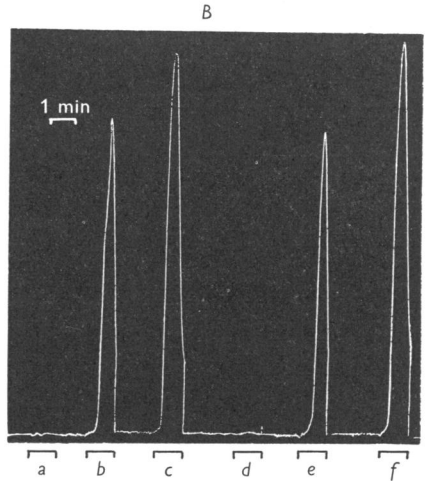


Fig. 3. Acid resistance (*A*) of kinin-forming factors in acid-treated human plasma and (*B*) of purified human serum kallikrein. Rat uterus. *A*. Kinin formation in PAP acidified for 15 min with N/6-HCl to pH 4·8 (*a*, *d*, *g*), pH 2·3 (*b*, *e*, *h*), and pH 1·2 (*c*, *f*, *i*). The PAP was re-neutralized and diluted 10 times at $t = 0$. All doses = 0·1 ml.; their times of administration are shown below the brackets. (*j*) = PAP acidified for 15 min with N-HCl to pH 0·5; 5 times diluted; 0·4 ml. at 45 min. (*k*) = 0·1 ml. of (*j*) + 0·1 ml. of PAP incubated for 3 min and applied at 50 min. *B*. (*a*) = 0·1 ml. of PAP; (*b*, *c*) = PAP incubated at room temp. with 0·02 u./ml. human serum kallikrein; 0·1 ml. applied at 3 min (*b*) and at 8 min (*c*); (*d*) = human serum kallikrein; 0·005 Frey units; (*e*, *f*) = PAP incubated at room temp. with kallikrein 0·02 u./ml. which had previously been kept at pH 2 for 20 min at room temp.; 0·1 ml. applied at 3 min (*e*) and 8 min (*f*).

had been kept at pH 2·2 for 5, 15 and 30 min formed kinin at very similar rates.

A considerable part of the kinin-forming system survived heating to 100° C at pH 2 for 10 min. In plasma re-neutralized after 30 min heating at pH 2 kinin did not develop, but forming activity could still be detected on addition of intact plasma.

Since such resistance to acid seemed to constitute a clear difference from plasma kallikrein, which is usually described as very labile at pH 1·8–2·0, acid treatments of various intensity and temperature were applied to two preparations of human and hog serum kallikrein. It was found that both contained a factor which formed kinin in intact human plasma and which was very stable at pH 1·8–2·0 (Fig. 3*B*). Acid-treated human kallikrein was also tested on the rat and cat blood pressure and it was found that the depressor effect was reduced by this treatment to a larger extent than the kinin-forming potency.

The relationship of the kinin-forming activity in acid-treated plasma to contact-responsive factors was examined by testing the effect of glass on acid-treated plasma. The brief contact (e.g. 1–2 min with ballotini, or 4–5 min in a glass vessel), which produces large amounts of kinin in non-acidified PAP, did not accelerate the slow spontaneous development of kinin in acid-treated plasma. Indeed, the brief exposure to the large surface area of ballotini reduced the subsequent spontaneous kinin formation in acid-treated plasma, showing that a significant proportion of forming factors and/or substrate had been removed by the glass. Much longer contact (e.g. 15–20 min rotation with ballotini) was needed to enhance the development of kinin or of the forming activity slightly above that which developed spontaneously during the same period. The dilution resulting from the acidification was not responsible for this deficiency. since non-acidified PAP, 5 times diluted, responded very well to glass.

In contrast to this lack of response to clean glass, acid-treated plasma was readily activated by ballotini which had previously been coated with non-acidified plasma. The activating factor which these ballotini had obtained from non-acidified plasma was not removed from the glass surface by thorough washing with distilled water or 0·9 % NaCl solution, but it could be eluted by alkaline solutions or by 7 % NaCl solution. The factor was in this respect very similar to HF (Waaler, 1959; Margolis, 1960). Plasma preparations which contained HF, but had lost component A or kallikrein ((A + B)-dep; heated plasma, see p. 505) also supplied effective coating (Fig. 4*a* and *c*). Ballotini previously treated with HF-deficient Hageman-trait plasma did not induce kinin formation in acid-treated plasma (Fig. 4*e*). The findings suggested that acid-treated plasma was activated by adsorbed HF, and that no other factor was required.

Thus, in terms of Margolis's scheme, the deficient response of acid-treated plasma to contact seemed to be due to its lack of functioning HF. This in turn would mean that the spontaneous kinin formation occurring in such plasma did not require HF. However, acid-treated Hageman-trait plasma formed only negligible amounts of kinin, though all factors (except HF) required for kinin formation by contact were present. HF is therefore essential for spontaneous kinin formation in acid-treated normal plasma. It is evident that this treatment does not abolish the enzymic functions of HF, but may impair its adsorptive properties. Alternatively, acid treatment may damage an intermediate factor involved in the rapid reaction between adsorbed HF and component A. This possibility could not be excluded by the present tests.

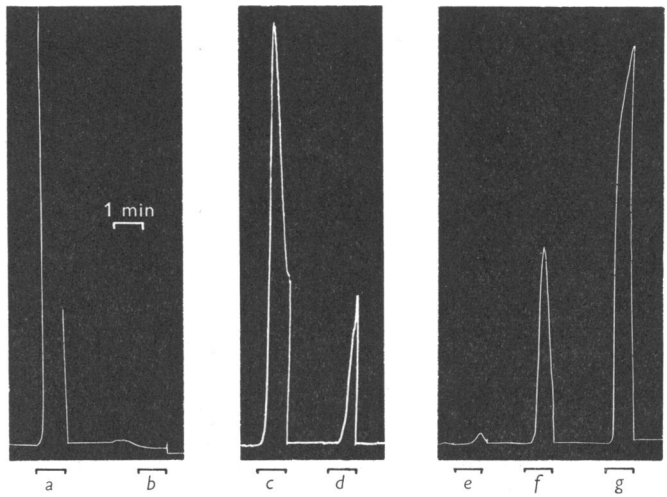


Fig. 4. Evidence that acid-treated PAP is activated by adsorbed HF. Rat uterus. All doses 0·1 ml., tested at the specified times after re-neutralization. Samples treated with glass were tested within 10 sec after separation from the ballotini. (*b*, *d*, *f*) = controls with acid-treated PAP, 10 times diluted, tested at 12 min to show kinin formed spontaneously. (*a*) = (*b*) activated at 5–7 min by ballotini coated with HF from (A + B)-dep.; (*c*) = (*d*) activated at 5–7 min by ballotini coated with HF from heated PAP (56° C for 3 hr); (*e*) = (*f*) rotated at 5–7 min with ballotini coated with Hageman trait (HF-deficient) plasma; (*g*) = (*f*) activated at 14–16 min by ballotini coated with HF from heated plasma.

Influence of previous exposure to glass

The effect of this on kinin formation after acid treatment was examined.

Activating contact. Forming factors and kinin activated in plasma by contact were preserved by acid treatment. PAP was exposed to ballotini 1 g/ml. At the height of activation acid was added. After re-neutraliza-

tion high concentrations of kinin and of forming activity were found which, at room temperature, continued to increase for approximately 4–5 hr.

Depleting contact. Plasma samples previously treated with glass according to the method of Margolis (1960) to achieve B- or (A + B)-depletion, still responded to acid with kinin formation, although non-acidified portions of these plasmas behaved as described by Margolis (1958) for B-dep or (A + B)-dep, respectively. The more extensively a plasma had been pre-treated with glass, the slower was the rate of kinin formation after acid treatment. Forming activity could be demonstrated in such plasmas several hours before the appearance of kinin. Brief activating contact with glass, immediately before acid treatment, considerably accelerated the development of kinin and of forming activity, even in plasmas which had previously been very thoroughly depleted.

The purified preparations of human and hog serum kallikrein resembled the kinin-forming agent in acid-treated plasma, in that they also formed kinin more slowly in B-dep than in the corresponding PAP.

Formation of kinin in acetone-treated plasma

In plasma precipitated with cold acetone (Lassen, 1958) and then redissolved in buffer solution, the spontaneous kinin formation dependent on HF, the response to activating contact, and the effect of trypsin inhibitors were very similar to those in acid-treated plasma. The inactivators of kinin-forming activity and of kinin were less completely destroyed than in acid-treated plasma.

Since a very similar precipitation with acetone has been recommended for obtaining the acid-labile plasma kallikrein (Frey *et al.* 1950, p. 111) it was of interest to see how acidity would affect kinin formation in Lassen's preparation. Its kinin-forming potency was not reduced by exposure to pH 2 for 15 min.

Kinin-forming properties of chloroform-treated plasma

On completion of Tagnon's (1942) procedure, i.e. 25 hr after incubation with chloroform, human plasma was free, or almost free, from kinin, but contained kinin-forming activity which could be demonstrated by incubating it with untreated PAP. Contact with ballotini enhanced the kinin-forming activity in chloroform-treated plasma 25 hr old. All kinin-forming activity in chloroform-treated plasma was inhibited by 1 mg SBTI/ml. or 10 mg LBTI/ml., which showed that this activity was not due to the esterase described by Austen (1960); OTI 20 mg/ml. had no effect. Acidification to pH 2 for 15 min caused little or no reduction of kinin-forming activity. Clean glass beads adsorbed from the preparation a coating which enabled them to activate Hageman-trait plasma. When

kept at 4° C the kinin-forming potency of a preparation was fairly stable and changed little during 2–3 days.

The effects of the treatment could now be summarized as follows: chloroform destroyed the inhibitors of the kinin-forming factors and thus allowed and perhaps promoted the activation of the latter in stable form. The active factors formed kinin, using up the substrate. The formed kinin was destroyed by the kininase which had not been abolished by chloroform.

Plasma kinin formation after separation of the euglobulin fraction

The precipitated and redissolved euglobulin fraction of human plasma, and the fraction remaining in the supernatant, were examined in the same way as the previous preparations. A kinin-forming system whose spontaneous activity required HF, and which also responded to adsorbed HF, was detected both in the euglobulins and in their supernatant. Inactivating factors were also present in both fractions. The supernatant contained about 4–5 times more kinin-yielding substrate than the euglobulins. Exposure to pH 2, or precipitation with M-NaCl at the same pH, did not significantly reduce the forming activity.

Kinin-forming properties of plasma heated at 56 and 61° C

Plasma heated at 56° C. After heating for 3 hr at 56–57° C human plasma regularly contained moderate levels of kinin, which decayed very slowly, and usually could be removed by dialysis at 4° C against 0·9 % NaCl solution. A remnant of inactivating factors could be detected in most samples. The kinin-forming properties of heated plasma suggested that component A was damaged by exposure to 56° C for 3 hr to a much greater degree than HF and kinin-yielding substrate (including component B). Thus, ballotini did not induce kinin formation in heated plasma (Fig. 5*a*, *b*), but they adsorbed from this plasma HF which enabled them to activate Hageman trait plasma deficient in HF (Fig. 5*c*). The substrate in heated plasma responded to the kinin-forming factor activated in unheated plasma by contact (*d*, *e*), acetone (*f*, *g*) and chloroform (*h*, *i*). When applied to heated plasma directly, acid, acetone or chloroform did not induce kinin formation. All these deficient responses of heated plasma could be corrected by mixing it before activation with plasmas which contained component A, but lacked other constituents necessary for normal kinin formation. Thus heated plasma could be corrected by B-dep or by Hageman trait plasma.

The findings suggested that all these modes of activation required at least two different forming factors. The following experiment shows that

both these factors are adsorbed on to silica surfaces. PAP heated at 56° C for 3 hr was rotated with ballotini 1 g/ml. for 3 min. No kinin was produced since heated plasma lacks component A (Fig. 6*a*). The ballotini were washed in the usual way and then rotated for 3 min with Hageman-trait plasma. Owing to the HF obtained from the heated plasma, these ballotini induced normal kinin formation in the HF-deficient Hageman trait plasma (Fig. 6*b*). The ballotini were then washed again and applied

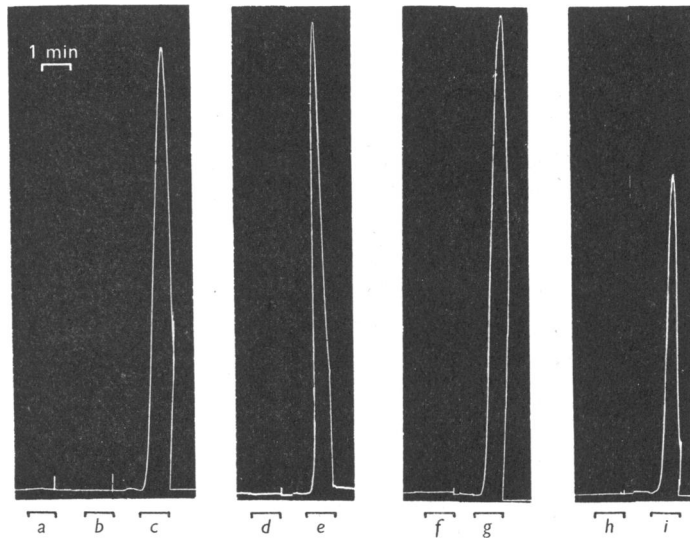


Fig. 5. Properties of PAP heated at 56° C for 3 hr. Rat uterus. (*a*) = 0·1 ml. of heated PAP; (*b*) = 0·1 ml. of (*a*) after 3 min rotation with ballotini; (*c*) = 0·1 ml. of Hageman-trait plasma after 3 min rotation with the ballotini from (*b*); (*d*) = 0·1 ml. of 3 times diluted (B)-dep after 3 min activation by ballotini; (*e*) = equal parts of (*a*) and (*d*), 0·15 ml. tested after 3 min incubation; (*f*) = acetone-treated (B)-dep, 5 times diluted, 0·1 ml. tested at 40 min; (*g*) = equal parts of (*a*) and (*f*), 0·15 ml. tested after 3 min incubation; (*h*) = 0·1 ml. of $CHCl_3$-treated plasma, 5 times diluted; (*i*) = equal parts of (*a*) and (*h*), 0·15 ml. tested after 3 min incubation. For details see text.

for 3 min to another portion of heated plasma. Intense kinin formation was now seen (Fig. 6*c*). This showed that the ballotini had adsorbed from HF-deficient plasma an activating factor which was not present in heated plasma. The efficacy of the coating acquired by the ballotini during the passage first through heated plasma and then through HF-deficient plasma was not due to double coating with one and the same factor: ballotini which had been immersed twice for 3 min in two portions of HF-deficient plasma did not activate a third portion of HF-deficient plasma (*d*); a similar passage through two portions of heated plasma did not enable

ballotini to activate a third portion of heated plasma (*e*). Ballotini immersed first in HF-deficient plasma and then in heated plasma did not induce kinin formation when added subsequently to another sample of heated plasma (*f*). It was unlikely that kinin adsorbed from the activated Hageman-trait plasma in test (*b*) contributed to the positive response of heated plasma in test (*c*), since ballotini immersed in B-dep (without producing detectable kinin; see *g*) also activated heated plasma (*h*).

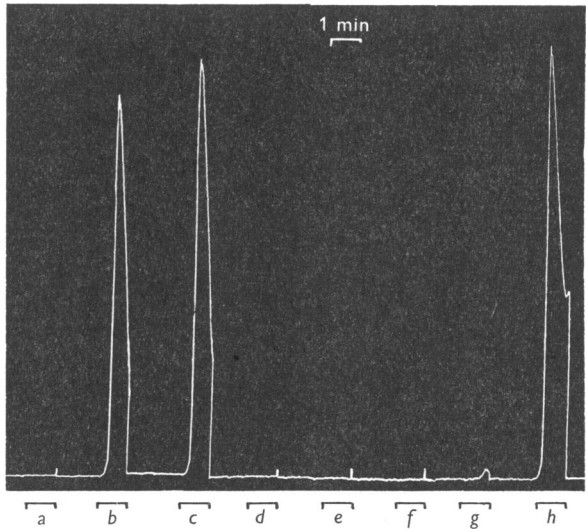


Fig. 6. Adsorption on to glass of HF from plasma heated at 56° C for 3 hr (deficient in component A), and of component A from Hageman trait plasma (deficient in HF). Tests (*a–h*) are described in the text. All doses 0·1 ml.

Since the factor abstracted from Hageman-trait plasma corresponded functionally to Margolis's component A, an attempt was made to use its adsorption on to HF-coated ballotini as a means of differentiating it from plasma kallikrein. Ballotini coated with HF from heated plasma were bathed in solutions of purified human plasma kallikrein. After thorough rinsing they were applied to another sample of heated plasma, in which they formed considerable amounts of kinin. Clean ballotini immersed in the same way in this solution of purified kallikrein did not activate heated plasma. Adsorbed HF thus facilitated the adsorption both of component A and of plasma kallikrein.

On the other hand, it would seem that in glass activation not only may HF itself be active in the adsorbed state, but that a small fraction of contact-activated HF remains in (or returns into) solution, particularly when the activating surface is not very large. This is suggested by the finding that small concentrations of a soluble kinin-forming factor can

be detected in plasma heated at 56° C after its activation in a glass vessel, but not after activation with ballotini.

Plasma heated at 61° C. Plasma heated at 61° C for 15–30 min, lost all endogenous kinin-forming potency. Margolis's (1958) finding that such plasma does not respond to glass, and does not coat glass with competent HF or any other kinin-forming factor, was confirmed. Acid or acetone treatment was not followed by spontaneous kinin formation. The response of Hageman-trait plasma to glass, acid or acetone was not corrected by PAP previously heated at 61° C.

Plasma heated at 61° C responded well to the same kinin-forming factors which were active on plasma heated to 56° C for 3 hr. It was clear from this that a considerable proportion of substrate (including component B) survived heating to 61° C. Remaining inactivating factors could be abolished by acid or acetone treatment, without reducing the response of the preparation to kinin-forming factors. Thus, by combining these treatments, inert yet activatable substrate could be obtained.

DISCUSSION

The present experiments have shown that four different methods (treatment with acid, acetone or chloroform and separation of the euglobulin fraction), designed to remove from human plasma the inhibitors of the fibrinolytic system, also removed plasma constituents which oppose intrinsic kinin formation. Moreover, provided the plasma contained HF active kinin-forming factors appeared after each of these treatments.

Before it is accepted that these factors belong to the fibrinolytic system it should be remembered that practically identical applications of acetone, and chloroform have been used to obtain plasma kallikrein (Frey *et al.* 1950, pp. 107, 112). Also, in chloroform-treated plasma, kinin-forming activity attained its peak after 24 hr, whilst the potency of plasmin continues to grow for several days (Christensen, 1946; Austen, 1960). Finally, after separation of the euglobulin fraction, the HF-dependent kinin-forming factors and their inactivators are, like the plasma kallikrein system (Webster & Pierce, 1960), present both in the euglobulins and in the supernatant. In contrast, fibrinolytic factors are found only in the euglobulin fraction and their inhibitors only in the supernatant (Norman, 1958).

On the other hand, the present experiments suggest that HF initiates kinin formation through factors whose resistance to acid is as great as that of plasmin (Müllertz, 1955), and far greater than that reported for plasma kallikrein (Werle & Maier, 1952; Bhoola, Calle & Schachter, 1960) and for permeability factor (Mill, Elder, Miles & Wilhelm, 1958). However,

differentiation of intrinsic plasma kinin-forming factors by their susceptibility to acid seems of limited value, since purified preparations of plasma kallikrein also contain an acid-resistant factor.

It would, therefore, be premature to attribute the observed kinin formation to plasmin. Before this can be done it is necessary to correlate this formation with at least one of the recognized enzymic activities of the fibrinolytic system.

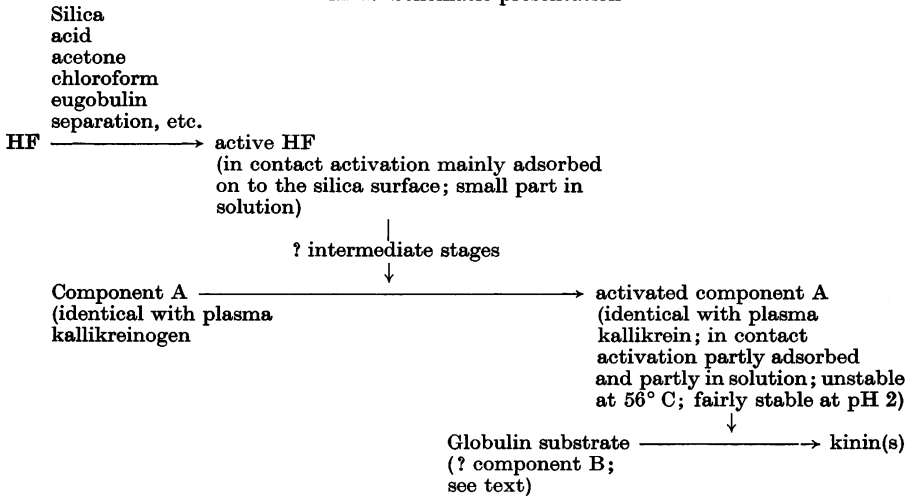
No fundamental difference between the kinin-forming factors activated by acid, acetone, chloroform, or by separation of the euglobulin fraction could be detected. A particularly important common feature was the dependence of the kinin-forming process on the presence of HF, which could be established for acid- or acetone-treated plasma, and for euglobulins. However, experiments with plasma heated at 56° C for 3 hr suggest that HF by itself is not sufficient for normal kinin formation in human plasma, but that a factor corresponding functionally to Margolis's component A is also required. Margolis defined this component as the soluble kinin-forming activity induced in plasma by contact, in distinction to HF, which he believed to be active only when adsorbed on to a surface. The experiment shown in Fig. 6, however, strongly suggests that activating surfaces also adsorb functionally significant amounts of component A. Similar conclusions can be drawn from some recent work by Margolis (1960). Moreover, the present experiments suggest that after various activating treatments, including activation by contact, HF may be active not only when adsorbed on to a surface, but in solution as well. This is supported by the finding that HF is required for the activation of plasma kallikreinogen by acetone (Webster & Ratnoff, 1961) and by several studies on the clot-promoting actions of HF (Ratnoff & Rosenblum, 1958; Ratnoff, Davie & Mallett, 1961). Component A and HF could, therefore, not be clearly distinguished by their adsorption characteristics. However, the present experiments suggest that these two factors can be distinguished by their resistance to heat at 56° C, component A being less stable than HF.

The lability of component A at 56° C is one of several features which this factor shares with plasma kallikrein (Werle, 1960). Other common properties are the similar resistance to acid and the adsorption on to HF-coated glass. B-depletion of the substrate plasma influences in a similar manner the kinin-forming effect of the contact-induced factor and of purified plasma kallikrein. Thus, the present study does not reveal any difference between these two kinin-forming factors. No direct comparisons with Permeability Factor were performed. This factor has not yet been clearly distinguished from plasma kallikrein.

The finding that, after the four plasma treatments which abolish plasmin

inhibitors, kinin was formed even if the plasma had previously been exposed to glass for B or (A + B)-depletion, could be raised in objection to the view that the observed kinin formation was due to the same factors which respond to activating contact. However, the discrepancy may be explained by the difference between untreated whole plasma, as used in Margolis's work (1958, 1960), containing inactivating as well as forming factors, and the plasma preparations used in the present work in which the inactivators had been abolished. In whole plasma, kinin would appear only if the rate of formation significantly exceeded the rate of inactivation. Similarly, the potency of kinin-forming factors would depend on the ratio of the rates of their activation and inhibition. 'Depletion' in these circumstances may merely mean that the velocities of the forming reactions,

TABLE 1. Schematic presentation



determined by the remaining concentrations of enzyme(s) and substrate(s) are too low to achieve detectable levels of kinin sufficiently rapidly. In the absence of inactivating agents the slower formation resulting from much lower concentrations of forming factors and substrate can readily be demonstrated. The difference between intact and glass-depleted plasma may therefore be merely quantitative, and a separate component B need not be postulated. This component can simply be regarded as that fraction of the substrate (kininogen) concentration in plasma, without which the rate of kinin formation by the glass-activated component A (or kallikrein) available in human plasma, becomes too low to overcome the normal plasma concentrations of inhibitors and/or inactivators.

The reported findings are compatible with the schematic presentation of intrinsic kinin formation in human plasma given in Table 1. It is an

extension and modification of the scheme suggested by Margolis (1958) for the activation of plasma by contact with silica surfaces. The possible role of the fibrinolytic system has not been considered in this scheme. This question is examined in the following paper.

SUMMARY

1. The kinin-forming properties of human plasma treated by four methods which are known to remove the inhibitors of the fibrinolytic system were examined. After each of the four methods plasma formed kinin without any further activating procedure.

(*a*) Exposure of plasma to pH 2 for 15 min destroyed kininase and the inactivators of kinin-forming activity. The amount of kinin formed in 1 ml. of plasma which had not been exposed to glass was equivalent to 1–10 μg of synthetic bradykinin. The yield was reduced by previous contact with glass beads. Normal kinin formation required Hageman factor (HF) and a factor which corresponded to component A (Margolis, 1959) and could not be distinguished from plasma kallikrein. Glass-activated kinin-forming factors, present in plasma at the moment of acidification, were preserved after acid treatment.

(*b*) Very similar, and probably identical, kinin-forming factors were found in plasma first precipitated with cold acetone and then redissolved in buffer solution, in chloroform-treated plasma, and in the separated euglobulins and their supernatant. Acetone destroyed the inactivators of both the forming activity and of kinin, chloroform only those of the forming activity.

2. Plasma heated at 56° C for 3 hr contained HF and kinin-yielding substrate. It lacked a factor which corresponded to component A. After brief heating at 61° C only substrate was present.

3. During contact activation a part of component A was adsorbed on to glass, together with HF. Adsorbed HF also facilitated the adsorption of plasma kallikrein. HF appeared to be active not only when adsorbed, but in solution as well.

I am very grateful to Professor C. A. Keele for his valuable advice and active interest in this work. Discussions with Drs D. F. Elliott, G. P. Lewis and M. E. Webster greatly helped me. My thanks are due to Mr F. J. Haydon and Miss Doreen Watson for technical assistance, to Mr M. Turney for photographic work, to Dr D. F. Elliott for synthetic bradykinin, and to Dr J. Margolis for Hageman trait plasma.

This work was done during the tenure of research grants generously given by the Empire Rheumatism Council. The studies reported in this and the following paper formed a part of the work submitted to the University of London in a thesis for the degree of Ph.D. (Eisen, 1961).

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