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A HIGHLY PURIFIED KININ-FORMING ENZYME FROM HUMAN PLASMA

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Abstract—An enzyme capable of liberating kinin has been isolated, in a highly purified form, from human euglobulins. It has some properties in common with each of the various kallikreins but, unlike them, it liberates from plasma a new, but so far unidentified, kinin instead of bradykinin, lysyl-bradykinin or methionyl-lysylbradykinin.

THE spontaneous liberation of kinins in ammonium sulphate precipitates of blood plasma has been studied by several groups of workers, with somewhat contradictory results. Habermann and Okon[1] treated the precipitated pseudo-globulins of ox plasma with HCl by the method of Gaddum and Horton[2] and, on neutralising, obtained bradykinin as the principal product, together with a small amount of a substance which was thought to be kallidin. However, Elliott[3] found that a slightly different acid treatment resulted in the "activation of an enzyme system capable of forming kallidin, bradykinin and perhaps other polypeptides with similar properties". One of the latter polypeptides has since been isolated and identified as methionyl-lysyl-bradykinin.[4] By contrast Armstrong and Mills[5] observed that, if the treatment with HCl was omitted, a euglobulin precipitate from human plasma formed two kinins, but neither of these was bradykinin or kallidin. In an effort to explain these discordant results an attempt has now been made to isolate and characterise the essential components of the reactions taking place in the third of these systems.[5] The purpose of this paper is to describe the preparation from human euglobulin of a highly purified kinin-liberating enzyme which has been distinguished from plasma kallikrein. These experiments have given no information about the nature of the precursors or mode of activation of this substance, and it will therefore be provisionally referred to by the non-committal title of "kinin-forming substance" (KFS).

MATERIALS AND METHODS

Blood

Human blood, containing 4–6 i.u./ml of heparin, was withdrawn from a heart–lung machine into siliconed bottles. It was stored at 4° until it was made available for use about 4 hr later. The storage bottles were coated with silicone MS 1107, which was applied as a 2% solution in acetone and polymerised by heating to 150° for 15 min.

Buffer solutions

The following weights of salts were dissolved in distilled water to give 1 l. of solution: *A* (pH 5·3); 0·625 g Na_2HP0_4 and 0·385 g citric acid.

B (pH 5·8); 14·04 g $NaH_2PO_42H_2O$ and 1·42 g Na_2HPO_4.
C (pH 7·5); 0·1329 g $NaH_2PO_42H_2O$ + 0·63 g Na_2HPO_4 + 8·36 g NaCl.
D (pH 7·5); 0·31 g $NaH_2PO_42H_2O$ + 1·47 g Na_2HPO_4 + 19·5 g NaCl.
E (pH 8·6); 12·55 g Sodium diethyl barbiturate + 1·715 g diethyl barbituric acid.
F (pH 6·5); 3·517 g $NaH_2PO_42H_2O$ and 1·342 g Na_2HPO_4.

Chromatography

For preparative experiments a column 28·5 cm × 3·6 cm of DEAE-cellulose (Whatman DE 50) was used. This was equilibrated against buffer B before use and was regenerated afterwards by washing with 1 N NaOH, water and buffer B.

Electrophoresis

The progress of the successive stages of the preparation has been followed by electrophoresis, on Whatman No. 3MM paper in buffer *E*, for 18 hr at a current of 1 mA per in. The active substance was located by cutting strips 1 cm wide from the paper, extracting them with water and assaying by the method described below.

At the final stage of the isolation of the enzyme, a preparative electrophoresis was performed under the same conditions but using buffer *F* as medium.

Method of assay

The amount of kinin liberated from human plasma under standard conditions has been used as a measure of the activity of the enzyme preparations. The detecting organ was the isolated rat uterus, suspended in a 10 ml bath of Ringer–Locke solution, the contractions induced by kinin being recorded by a lever writing on a kymograph. The plasma used as substrate was obtained from blood that had been drawn in a siliconed syringe, and was handled in polythene or siliconed apparatus.[6] An equal volume of the preparation to be tested was added to plasma and, after 1½ min incubation at room temperature, 0·1 ml of this mixture was introduced into the organ bath where it remained in contact with the muscle for 1 min. The contraction observed was compared with that produced by known concentrations of crystalline, synthetic bradykinin.

As an approximate measure of the specific activity of the preparations, a figure of merit has been derived from comparisons with a reference preparation of enzyme. The latter comprised a batch of material reserved at Step 4 of one isolation experiment (see below), and stored either as solution in Buffer *D* or as freeze dried solid at −15°. At each stage of isolation a test sample was progressively diluted until, when assayed, it produced a response equal to that induced by a weak preparation of the reference enzyme. The ratio of this critical dilution to the optical density of the undiluted sample at 280 mμ defines the figure of merit of the sample. The earlier stages of the purification were allowed to exhaust their spontaneous kinin-forming ability by dialysis against 0·9% NaCl solution overnight, before being submitted to the assay.

Isolation of the enzyme

The method is outlined in Fig. 1. It will be seen that the earlier manipulations are carried out at about pH 5 in order to reduce the spontaneous activation to a negligible rate. Although both the fractions produced by dialysis at this pH were able to form kinin spontaneously when neutralised, the precipitate was found to be the most convenient source of the enzyme and it is the preparation *via* this route that will be described.

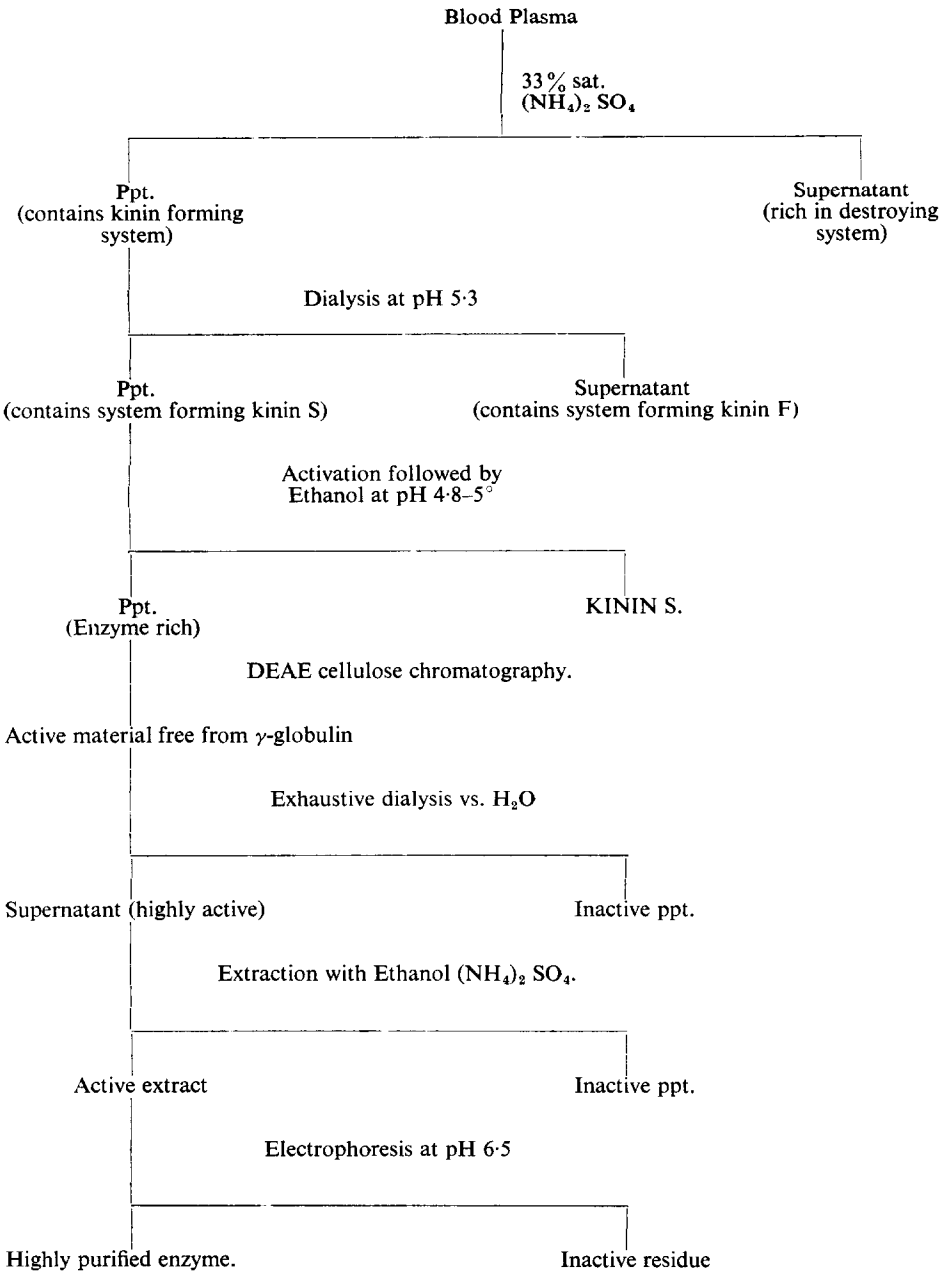

FIG. 1. Isolation of kinin-forming enzyme from human blood plasma.

Step 1. The blood was centrifuged for 30 min in siliconed bottles and the plasma removed through a polythene tube into a polythene bottle. The plasma was mixed with 0·5 vol. of neutral, saturated ammonium sulphate solution, and the precipitate centrifuged off in polythene buckets. After washing with 33% saturated ammonium sulphate, the damp solid was stored at −15° until required. A solution of this precipitate had a figure of merit of about 1·0.

Step 2. A solution of 100 g of the frozen precipitate in 200 ml of buffer *A* was dialysed for 24 hr against two 6 l. vol. of the same buffer. This procedure brought about the formation in the dialysis bags of a precipitate which was rich in kinin-forming substance but contained relatively little kininase. It was collected by centrifugation.

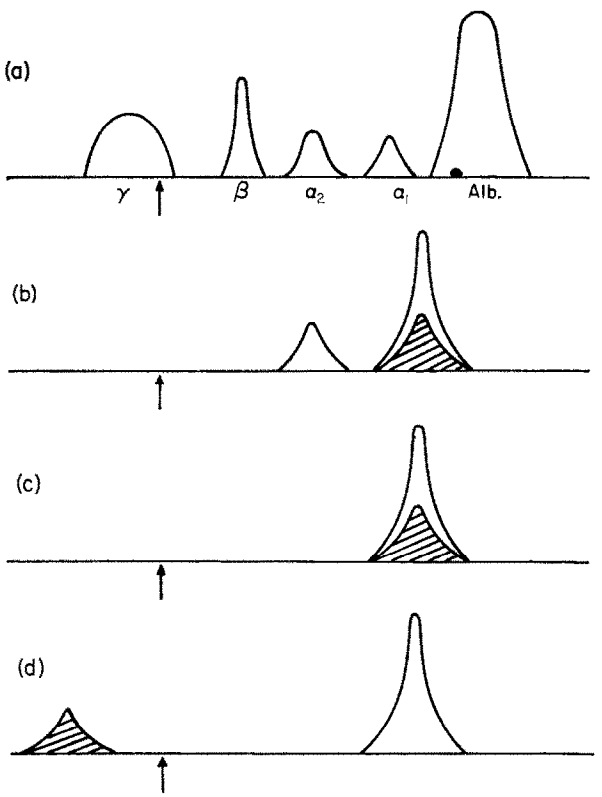

FIG. 2. Paper electrophoresis of solutions obtained at different stages in the isolation of KFS. The representation is diagrammatic.

The points of application are indicated by arrows. The inactive proteins were located by staining with Light Green, and the position of KFS, shown by the shaded areas, was found by the kinin-liberating ability of eluted strips.

The solvent used in experiments (a), (b) and (c) was Buffer *E*, pH 8·6, in which migration was anodic.

(a) the original blood plasma; the components are identified according to the usual nomenclature.

(b) Step 4—the active supernatant. Kinin-forming activity migrates with the leading peak which moves with a rate between that of albumin and α-globulin.

(c) The highly purified stable preparation of KFS (Step 5).

(d) Separation of KFS from the major impurity of Step 5 by electrophoresis in Buffer *F* at pH 6·5.

Step 3. The precipitate produced in stage 2 was dissolved in 150 ml of 0·9% NaCl solution and the pH raised to about 8 by the addition of sodium bicarbonate. Serial assays showed that the resulting liberation of kinin ceased after about 3 hr, at which time sufficient citric acid was added to reduce the pH to 4·8. The solution was then cooled to −5° and 0·33 vol. of ethanol, at −5°, was slowly added with continuous stirring. The precipitate formed was centrifuged off at −5°, dissolved in 100 ml of 0·9% NaCl and dialysed against buffer *B*. This solution had a figure of merit of about 12 and constituted the crude enzyme solution used in the next stage.

Step 4. 60 ml of the solution from stage 3 were run into the column of DEAE-cellulose, which was then developed chromatographically with the following solvents: (1) 1·0 l. buffer *B*, (2) 1·5 l. buffer *C*, (3) 1·5 l. buffer *D*. Displacement of the enzyme from the column began when the NaCl concentration of the effluent reached about 0·18 M. The active part of the effluent was dialysed against distilled water, reduced in bulk in a rotary evaporator, and then dialysed exhaustively against distilled water. This brought about the formation of an inactive precipitate which was discarded. The active supernatant had a figure of merit of about 625 and two main components were distinguishable by paper electrophoresis (Fig. 2b).

Step 5. The active solution from stage 4 was converted into a two phase system[7] by the addition, to each volume of solution, of 3 vol. of ammonium sulphate solution (40 g in 100 ml water) and 2 vol. of ethanol. On shaking, the kinin-forming substance passed almost completely into the ethanol rich phase, whereas much inactive material either remained in the lower phase or was precipitated.

The upper phase was dialysed against distilled water and concentrated in a rotary evaporator to small bulk. Paper electrophoresis (in buffer *E*) and ultracentrifugation both revealed only one component in this solution (Fig. 2c), which had a figure of merit of about 3300.

Step 6. The removal of the remaining major impurity has been unexpectedly difficult. The most successful method of preparing a highly purified material has been the electrophoresis of the product of stage 5, on paper in buffer *F*. Under the conditions described in the Methods section the kinin-forming substance moved towards the

TABLE 1. SPECIFIC ACTIVITY AND YIELD OF KININ-FORMING SUBSTANCE AT VARIOUS STAGES OF ITS PURIFICATION

Purification stage	Recovery (per cent)	Figure of merit
1	—	1
2	75–80	—
3	65–70	12
4	80–85	625
5	25–30	3300
6	low, variable	15,000

cathode, whereas the migration of the residual impurity was anodic (Fig. 2d). The active material was recovered from the paper by elution with water or Ringer–Locke solution. The material obtained in this way was unstable; recoveries were rather low, and the kinin-forming activity often vanished within a few hours.

Recovery and specific activity

The recovery of kinin-forming activity at each stage of the separation is summarised in Table 1, together with the corresponding figures of merit. The recoveries are expressed as a percentage of the activity of the preceding stage, with stage 1 as base. The total recovery from plasma was about 15 per cent at stage 5 and, at the final stage, about 2 per cent. The final figure of merit is estimated to correspond to a level of 1–2 μg enzyme/ml of original plasma.

PROPERTIES

The kinin-forming substance, used in equal concentrations will bring about the liberation of kinin, not only in normal human plasma, but also in Hageman factor deficient plasma and in monkey, guinea-pig and rat plasmas. By contrast, cat plasma is inert. In none of these cases has the kinin formed been chemically identified.

When a solution of KFS was added to an equal volume of human plasma (collected by the method described in Ref. 6) to give a final enzyme concentration of 50 μg/ml, the uterus contracting activity liberated in 1·5 min at room temperature was equivalent to about 0·7 μg of synthetic bradykinin/ml mixture. By paper chromatography, however, the product of this reaction could be distinguished both from bradykinin and from kallidin.[5] Furthermore, it was easily destroyed by kininase and could thus be differentiated from methionyl-lysylbradykinin which is resistant to this treatment.[8] Unlike angiotensin it relaxes the rat duodenum.

High concentrations of the enzyme itself can induce contractions of the isolated rat uterus after a latent period which varies inversely with the concentration used. However, unlike the contractions stimulated by kinin, those induced by the enzyme are antagonised by soya-bean trypsin inhibitor (SBTI).

On intradermal injection in the rat, the KFE preparation increased capillary permeability, and the action was antagonised by SBTI but not by the antihistamine drug mepyramine.

Though unpurified KFE lysed both heated and unheated fibrin plates, the fibrinolytic activity of the Step 5 preparation was feeble on unheated plates and almost completely absent on heated ones.[9]

Stability

(*a*) *Effect of heat.* The highly purified substance obtained by electrophoresis appears to be unstable at room temperature, although the rate of decay has varied widely in different experiments. By contrast, the material from stage 5 of the isolation procedure, which contains one major impurity, is considerably more resistant to heating. Thus, the half-life of this preparation, in pH 8·1 phosphate buffer, is about 1 min at 100°, 4 min at 79° and 8 min at 69°. At 60° it can be heated for 1 hr without significant loss of activity.

(*b*) *Effect of pH.* The most pure preparation on which this could be studied was that from stage 5, which has been tested over the range of pH 2·5–10·2. The enzyme mixture was dialysed for 24 hr at room temperature, against the appropriate buffer solution and the residual activity measured. Citric acid–phosphate buffers were used up to pH 8 and carbonate–bicarbonate above this. The results are presented graphically in Fig. 3 which shows that this preparation is stable between pH 6·5 and 9.

pH of optimum activity

This has been determined both by the rate of kinin formation in the redissolved precipitate from Step 2, and by the hydrolysis of *p*-toluenesulphonyl-L-arginine methyl ester (TAME). In the former case the solution was divided into several parts which

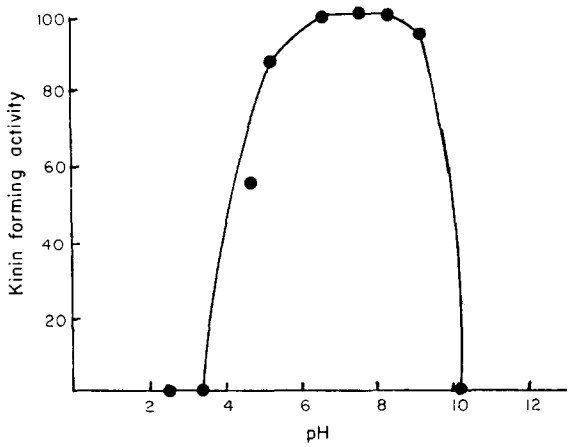

FIG. 3. pH stability of KFS.

The ordinate represents percentage of original kinin-liberating activity remaining after 24 hr treatment.

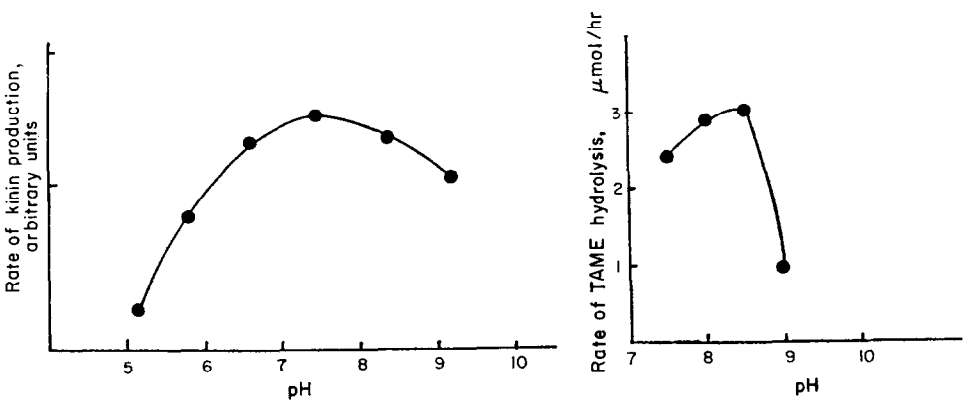

FIG. 4. pH of optimum activity of KFS for two substrates.

(a) Rate of kinin production during the auto-activation of the human euglobulin fraction from Step 2 of the isolation procedure.

(b) Rate of hydrolysis of TAME by the enzyme obtained from Step 5.

were then adjusted to the required pH by the addition of phosphate or bicarbonate buffer. After 7 min incubation at room temperature the kinin level in each mixture was assayed. As Fig. 4(a) shows, maximum activity in this system occurs at about pH 7·5. In the reaction with TAME the stable concentrate from Step 5 was used because the purified substance would decompose appreciably during the course of an experiment. The rate of digestion of 0·01 M TAME was measured by titration with

0·1 M NaOH in the Radiometer Type TTT 1a titrigraph. These experiments showed (Fig. 4b) that a maximum rate of 3·0 μmol TAME/h/mg active fraction occurred at pH 8·5. Since the preparation contained only 20–25 per cent active material the rate per mg of enzyme must be at least 12–15 μmol/h.

Electrophoresis

Because of its instability it has not been possible to test the homogeneity of the highly purified material either by electrophoresis or ultracentrifugation. However, electrophoresis on cellulose acetate paper, ("Oxoid") over a range of pH from 4–8 in a variety of buffers, produced only one band of active material which was almost coincident with a single band of an inert substance. Under these conditions the migration of both substances was anodic, with the isoelectric point falling between pH 4 and 4·5. On ordinary paper (Whatman 3MM) a quite different behaviour has been observed, viz. at all pH values from 9 to 5·5 the impurity, which is the major constituent of the mixture, migrates towards the anode with unchanged velocity. At pH 8·6 this substance moves with the speed of a plasma α-globulin. In this system, at pH 8–9, the active substance also migrates anodically at the same rate as the impurity, but at pH 6·5 or less it moves as a diffuse band in the opposite direction. No explanation has been found for this behaviour but, since it occurs in several different buffer mixtures, it appears to be connected in some way with the properties of the unmodified paper.

Thus, the electrophoretic behaviour of KFS on an inert support resembles that of the urinary, pancreatic or salivary kallikreins.[10,11]

Inhibitors

Soya-bean trypsin inhibitor, (SBTI) when added to the KFS in the proportion of 1 part to 20 of the stable preparation (W : W), will completely suppress its activity after an incubation of 15 min at 37°. If the incubation time was shortened or the inhibitor concentration lowered five fold, the enzyme was only partially inactivated. By contrast, the addition of a 5000 fold excess by weight of ovomucoid trypsin inhibitor caused no loss of activity.

Complete inhibition could be caused by incubation for 4 hr at 37° with 10^{-3} M diisopropylphosphorofluoridate (DFP) and a partial inactivation could still be produced if the concentration of DFP was reduced to 10^{-5} M.

An irreversible inhibition can also be brought about by hexadimethrine bromide (Poly-(NNN′N′-tetramethyl-N-trimethylene hexamethylene diammonium dibromide). Since this substance is adsorbed by glass, the measurements must be made in polythene vessels. Under these conditions the KFS is inhibited by an equal weight of hexadimethrine bromide after 5 min incubation at 20°.

DISCUSSION

Since the kinin-forming substance can hydrolyse TAME *in vitro* it possesses enzymic properties to which its ability to liberate kinin may plausibly be ascribed, and it may therefore be included with those enzymes which are collectively known as kallikreins. The most completely characterised preparation of human plasma kallikrein appears to be that extracted from acetone activated, outdated "bank" plasma.[12,13] However, this enzyme differs from KFS in the following two respects. When added to plasma,

kallikrein liberates bradykinin,[13] whereas KFS produces a different kinin that has provisionally been called "kinin E".[5] Secondly, kallikrein is not adsorbed by DEAE-cellulose at pH 7, but is readily adsorbed on CM-cellulose at pH 5.[12,13] By contrast, KFS acts in exactly the opposite way and its behaviour towards ion-exchange substances and on electrophoresis shows that KFS is negatively charged under conditions when kallikrein is positively charged (cf. Ref. 10). In this respect KFS is like the kallikreins of pancreas, saliva or urine, or the hog plasma enzyme of Werle and Trautschold[14] which is prepared by adsorption on reprecipitated casein. It is noteworthy also, that the latter enzyme was, like KFS, unstable when highly purified.

Although plasmin can also form kinin, it can be distinguished from KFS by the same properties that differentiate it from kallikrein, e.g. it will withstand boiling at pH 2 for 30 min,[15] whereas the latter will not. In particular, when a solution of KFS was made fibrinolytically equipotent with the plasmin liberated by a given solution of thrombolysin, KFS was 200 times more effective as a liberator of kinin. But when the solutions were made equal with respect to kinin-forming ability, the KFS had only 0·04 times the fibrinolytic activity of the thrombolysin.[9]

From this evidence it has been concluded that plasma contains at least three enzymes potentially capable of liberating kinin, and that the KFS may be identifiable with the enzyme studied by Werle. It may be significant therefore, that the euglobulin precipitation method of Milstone[16] has been reported[12] to separate kallikreinogen into two parts. One of these was precipitated with the euglobulins, while the other remained in the supernatant. When the zymogens were activated, however, the resulting enzymes could not be differentiated, but this may have been due to a lack of discriminatory power in the tests used.

At one stage of their treatment of acetone-activated plasma Webster and Pierce[13] extracted with DEAE-cellulose under the conditions used here to adsorb KFS, but, although this fraction contained an esterase[17] it did not exhibit the properties of kallikrein, nor was it inhibited by SBTI. This contrast with the behaviour of KFS has not been completely resolved, but it should be noted that Armstrong *et al.*[18] have commented on the feeble activation of dog plasma by glass, and we have now found that, as a substrate for KFS, "pre-active" dog plasma is only 5–7 per cent as effective as human plasma. Thus their failure to observe a significant fall in blood pressure on intravenous administration of their preparation to the dog may have led Webster and Pierce to underestimate its potential activity. Such a difference in behaviour towards the dog, has already been observed in the case of the salivary and pancreatic kallikreins of the pig.[19]

In its capacity to hydrolyse TAME with an optimum activity at pH 8·5, the kinin-forming substance is like the kallikreins of plasma, pancreas and urine (cf. Ref. 17). However, the inactivation by SBTI which serves to emphasise the similarity of KFS to plasma kallikrein, also distinguishes it from the pancreatic, salivary and urinary enzymes, which are unaffected by this inhibitor.

The diminished thermal stability of highly purified KFS, (which is like that reported of hog serum kallikrein[14] and also of the horse urinary enzyme[20]), suggests that although KFS is intrinsically rather labile, it exists in *activated* plasma as a more stable complex. However, although the partially purified preparation of KFS was still fully active after heating for 1 hr at 60°, the kinin-forming ability of *native* plasma is destroyed after only 30 min at this temperature. In the case of the kallikrein liberated

by trypsin, Werle[21] claims that heating destroys the enzyme precursor. This may also be true of the zymogen of KFS, but the possibility that the sequence of activation reactions is broken at a still earlier stage has not yet been eliminated.

Although little is known of the mechanism of kinin formation in ox pseudoglobulins, the available evidence is not inconsistent with the suggestion that whereas the active agent in the human euglobulins is KFS, that present in the ox pseudoglobulin may be plasma kallikrein. It may thus be significant that the preparation used by Elliott[3] was still able to form kinin although the acid treatment to which it was subjected would be sufficient to destroy any KFS that was present. However, the mechanism of kinin formation in the two systems cannot be fully explained until all the reactants in both have been identified.

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