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The isolation of human plasma prekallikrein

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Summary

1. The isolation of human plasma prekallikrein was achieved by fractionating human plasma on diethylaminoethyl cellulose (DEAE) in the presence of heparin.

2. Heparin was shown to inhibit the activation of prekallikrein during the isolation procedure.

3. The isolated prekallikrein fraction had some kallikrein activity which could be inhibited by diisopropylfluorophosphate (DFP) without affecting the ability of prekallikrein to be activated.

4. The prekallikrein obtained was functionally pure in that it had no kallikrein inhibiting or activating activity. It was not physico-chemically pure, the major contaminant being the immunoglobulin IgG.

Introduction

Plasma kallikreins are proteolytic enzymes in human and other mammalian plasmas that release vasoactive polypeptides termed kinins from a plasma substrate termed kininogen (Werle, 1955). These polypeptides are potent mediators of vasodilatation and increased vascular permeability, produce pain and influence the migration of leucocytes. Because of these properties, kinins have been proposed as possible mediators of the inflammatory response (reviewed by Kellermeyer & Graham, 1968).

Plasma kallikrein exists in plasma in the form of an inactive precursor, prekallikrein. Because of its role in releasing kinins, it is important to understand its mode of activation. It is activated when whole plasma is brought into contact with glass or similar surfaces, a process initiated by the activation of another plasma protein—Hageman factor (Margolis, 1960). At present, however, the exact sequence of events that leads to the activation of prekallikrein is not clear. One possibility is that Hageman factor first activates other enzymes such as plasmin or Pf/dil (Vogt, 1964 ; Mason & Miles, 1962) which then convert prekallikrein to kallikrein. Another possibility is that Hageman factor directly converts prekallikrein (Nagasawa, Takahashi, Koida, Suzuki & Schoenmakers, 1968). The availability of a functionally pure preparation of prekallikrein would be of considerable help in defining this sequence. The preparation should contain no kallikrein, prekallikrein activator or kallikrein inhibitor. The purpose of this paper is to describe a method for isolating such a preparation.

Other investigators have noted in the early stages of the purification of kallikrein the presence of prekallikrein which could be activated by glass and kaolin surfaces pretreated with plasma (Davies & Lowe, 1963 ; Colman, Mattler & Sherry, 1969).

Human prekallikrein has not been isolated, but rabbit and bovine prekallikrein have. Rabbit prekallikrein was isolated in the presence of hexadimethrine bromide, a substance that prevents the activation of Hageman factor (Eisen, 1964 ; Wuepper, Tucker & Cochrane, 1969). Bovine prekallikrein was isolated by the combined use of ammonium sulphate precipitation and DEAE and carboxymethyl (CM) cellulose ion exchange chromatography ; no inhibitor was used to retard the activation of prekallikrein (Nagasawa *et al.*, 1968).

The problem encountered in isolating human prekallikrein is its progressive activation during purification due to the necessary contact with foreign surfaces. To circumvent this problem in the present work, it was thought best to attempt the isolation in the presence of an inhibitor. Ideally, the inhibitor should maintain prekallikrein in its inactive state, but be separable from it during purification. Heparin in high concentration retards the activation of Pf/dil and kallikrein in whole plasma (Elder & Wilhelm, 1958 ; McConnell & Becker, 1966). Since it is strongly electronegative, it should bind tightly to DEAE whereas human prekallikrein, if it behaves like guinea-pig prekallikrein (Davies & Lowe, 1963), should bind weakly. Accordingly, heparin was used to inhibit prekallikrein activation and, using this substance, we succeeded in isolating a stable preparation of prekallikrein free from inhibitors and activators.

Methods

Plasma

Blood (4 parts) was taken into acid citrate dextrose (ACD) (1 part) in plastic Fenwal bags (Baxter) and centrifuged to separate the cells. The supernatant plasma was removed immediately and stored frozen at $-30°$ C until used, when it was thawed and poured into a siliconized vessel. When heparin was to be added, it was added at this stage, to give a final concentration of 100 units/ml. After the addition of heparin, all procedures were carried out in glass.

Siliconized glassware

Siliconized glassware was prepared by established methods (Ratnoff & Miles, 1964).

DEAE fractionation

DEAE batch procedures. DEAE-52 (Whatman), equilibrated with 0·025 M Tris-hydrochloride pH 7·6, was poured into a sintered glass Buchner funnel and allowed to settle. A filter paper was placed on top of the DEAE to stabilize the surface and plasma was added dropwise and then allowed to adsorb to the DEAE for 1 h before elution. The first peak of protein was eluted with 0·025 M Tris-hydrochloride pH 7·6 ; and the remainder was eluted by washing with 0·05 M Tris-hydrochloride pH 7·6 in 0·3 M sodium chloride until most of the protein was off and then by washing with 1·0 M sodium chloride. The effluent was monitored for pH, conductivity, protein concentration, and ability to increase vascular permeability and to release kinin from human kininogen before and after activation with coated ballotini. All fractionations were carried out at $+4°$ C. For details of individual runs, see **Results**.

DEAE chromatography. DEAE chromatography was carried out by standard methods (Sober, Gutter, Wyckoff & Peterson, 1956). Columns 1×30 cm were packed under gravity pressure with DEAE-52 that had been equilibrated with 0·025 M Tris-hydrochloride pH 7·6. One ml samples of kallikrein solution were applied to the top of the column and allowed to adsorb for one hour. The material was eluted from the column with buffer pumped at 10 ml/h from a linear gradient device containing 150 ml of 0·025 M Tris-hydrochloride pH 7·6 in the first chamber and 0·05 M Tris-hydrochloride in 0·3 M sodium chloride pH 7·6 in the second. All operations were performed at +4° C.

Ultrafiltration

Ultrafiltration was carried out at +4° C by established methods (Craig, 1968).

Assay methods

Protein. During the fractionation, protein concentration was determined from optical density at 280 nm. In certain instances it was determined from the nitrogen content with Nessler's reagent (Meynell & Meynell, 1965).

Heparin. One lot of heparin (Boots Pure Drug Co. Ltd., Batch No. 13W, 160 international units/mg) was used for all experiments. Heparin was assayed by a modification of methods described by Jaques & Bell (1959) and Altescu (1963). Four ml of 80% phenol (80 g of phenol + 20 ml of H_2O) were added to 4·0 ml of unknown in a centrifuge tube. The tubes were capped, shaken vigorously and held overnight at room temperature. The protein and phenol were removed by centrifugation at 2,500 rev/min for 20 min at room temperature and all but 1·0 ml of the top aqueous layer was removed and dialysed against distilled water. The dialysed material (1·0 ml) was then mixed with 3·0 ml of 0·25% streptomycin (Glaxo Lab. Ltd.) at room temperature and the optical density of this solution read at 320 nm (ultraviolet) on a spectrophotometer. The heparin concentration of the unknown was determined from a standard curve.

The relation between heparin concentration and optical density was linear for concentrations between 2·5 and 32·0 units/ml and heparin concentration could be determined accurately as long as the plasma protein concentration was less than 2 mg/ml. At higher protein concentrations, the readings indicated more heparin than was present. The assay is not specific for heparin, but within the limits described it was satisfactory.

Kinin. Kinins were assayed on the isolated oestrus rat uterus using an auxotonic system (Paton, 1957; Eisen, 1963). Test solutions were applied to the uterus by adding them to 30 ml of de Jalon's solution at 30° C contained in the barrel of a syringe connected by a short piece of tubing to a 10 ml tube that held the uterus. A plunger was introduced into the barrel and the entire 30 ml gently injected into the preparation tube. The kinin in an unknown was determined by matching the height of contraction it produced with that of a known amount of synthetic bradykinin (Sandoz) giving an equivalent contraction. Results were expressed in nanograms of bradykinin equivalent. Test solutions were applied to the uterus at 6 min intervals and were alternated with the bradykinin standards.

Kallikrein. A single batch of substrate for the assay of kallikrein was used throughout the work. It was prepared by heating one litre of outdated ACD plasma

for one hour at 61° C. The precipitate that formed was separated by centrifugation, and the supernatant fluid dialysed against 0·01 M sodium phosphate in 0·15 M sodium chloride pH 7·5 and then frozen at −30° C until used. This substrate contained no detectable kinin or kininase.

For the kallikrein assay 0·4 ml of the unknown, which had been dialysed against 0·01 M sodium phosphate in 0·15 M sodium chloride pH 7·5, was incubated with 0·4 ml of substrate at 37° C for varying periods and the kinin content of 0·6 ml of this mixture determined.

Vascular permeability. The assay of permeability increasing activity was performed in guinea-pigs with pontamine blue in their circulation as described by Wilhelm, Mill, Sparrow, MacKay & Miles (1958) with certain modifications: because the solutions tested were weak, the injection volume was 0·2 ml instead of 0·1 ml; animals were treated with intravenous triprolidine (Wellcome) (100 μg base/kg body weight) to minimize and make more uniform the amount of blueing produced by needle trauma; and lesion size was measured 15 min after injection. The plot of mean lesion diameter against log concentration of the substance under test could be fitted with a straight line, from which the average dose of permeability factor that produced a 7·0 mm lesion could be determined. For the purposes of this paper, a blueing dose was defined as the amount of a permeability factor in a 0·2 ml injection volume that, on the average, induced a lesion 7·0 mm in diameter in the skin of the blued guinea-pig 15 min after injection. The amount of material in this blueing dose is about one-fifth the "effective blueing dose" described by Wilhelm *et al.* (1958).

Coated ballotini

Method of coating. Clean ballotini (grade No. 13, R. W. Jennings & Co.) were coated by rotating them with twice their volume of 1/50 human plasma in 0·15 M sodium chloride for 10 min at room temperature and washing them ten times with 0·15 M sodium chloride. Ballotini were exposed to kallikrein in a similar fashion, substituting a solution of kallikrein in 0·015 M sodium phosphate in 0·15 M sodium chloride pH 7·5 (14 kallikrein doses/ml—see below) for the 1/50 human plasma. Unless otherwise stated, the term "coated ballotini" refers to plasma coated ballotini.

Activation of substances with coated ballotini. All solutions for test were brought to pH 7·5 and ionic strength 0·15 before activation. One volume of inactive material was rotated with one-half volume of coated ballotini for 10 min at room temperature. The term "activation" in this paper refers to activation with plasma coated ballotini.

Inhibitors of kallikrein activity

Diisopropylfluorophosphate (DFP). The substance to be treated was incubated in 0·01 M DFP (kindly donated by Dr. B. C. Saunders) in 2% ethanol in 0·15 M sodium chloride for 3 h at 37° C, and then for 18 h at +4° C. The material was then dialysed extensively against 0·01 M sodium phosphate in 0·15 M sodium chloride pH 7·5. A control sample was treated in the same manner except that DFP was omitted.

Soyabean trypsin inhibitor (SBTI). SBTI (Worthington) was dissolved at a concentration of 10 mg/ml in a mixture of 0·5 ml sodium-potassium phosphate buffer (pH 8·0, ionic strength 0·2) and 4·5 ml of 0·15 M sodium chloride. This stock solution was kept frozen and suitable dilutions in 0·15 M sodium chloride were prepared for use.

Immunoelectrophoresis

Immunoelectrophoresis was carried out by established methods (Scheidegger, 1955).

Results

Definition of "kallikrein dose"

Because the term " unit " can be taken to imply the activity of a certain weight of an agreed standard preparation of plasma kallikrein, the term " dose " has been used in this paper to express amounts of human plasma kallikrein. The method we devised for measuring kallikrein doses was as follows.

Figure 1 exemplifies the plot of a series of curves obtained of kinin yield when graded amounts of a kallikrein preparation were incubated with the standard substrate for varying periods. Other batches gave similar sets of curves. By 36 min the yield of bradykinin equivalent released by all the amounts of kallikrein tested had reached a plateau. Under the conditions of these experiments, 30 ng bradykinin equivalent was the minimum that could be detected from one day to the next.

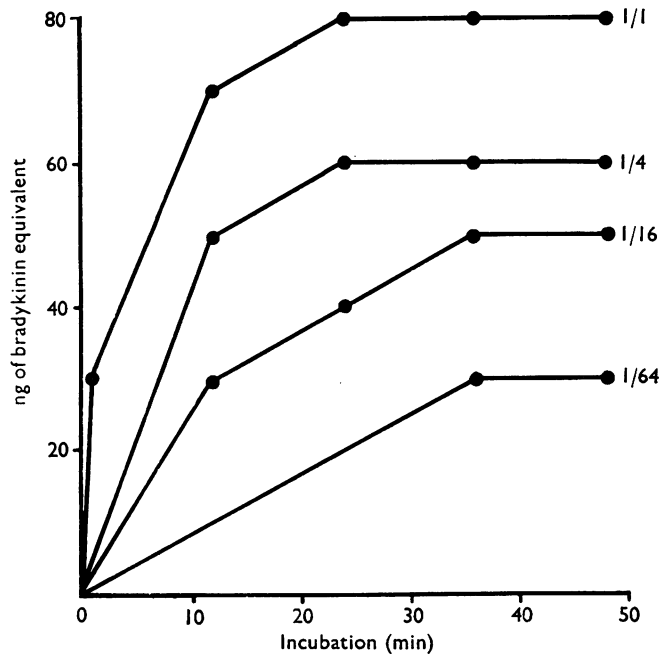

FIG. 1. Bradykinin equivalent released from standard substrate by graded dilutions (1/1, 1/4, 1/16, 1/64) of a kallikrein fraction at 37° C, pH 7·5, ionic strength 0·15 for periods up to 48 min.

Figure 2 demonstrates that when the ng of bradykinin equivalent released by kallikrein after 36 min is plotted against log dilution of kallikrein, the points can be fitted with a straight line between 30 ng and 80 ng of bradykinin equivalent. From this line, the amount of the kallikrein preparation in 0·3 ml that releases 30 ng of bradykinin equivalent can be determined. The values for this amount were found to be reasonably similar in repeated tests of the same enzyme preparation on different uterus preparations, a regression line being determined in each case. The coefficient of variation about the mean of these values was ±20%, both in the example cited and with all of a number of enzyme preparations examined in this way.

For the purposes of this paper, a " kallikrein dose " is defined as the amount of the kallikrein preparation in 0·3 ml that on the average releases 30 ng of bradykinin equivalent from 0·3 ml of standard substrate when incubated with that substrate for 36 min at 37° C at a pH of 7·5 and ionic strength 0·15. The concentration of kallikrein is expressed in kallikrein doses/ml. The prekallikrein content of a preparation is taken as the difference between the kallikrein content before and after activation with coated ballotini and is expressed in prekallikrein doses/ml.

Heparin inhibition of prekallikrein activation

The following test was made to determine the value of heparin as an inhibitor of the activation of prekallikrein during fractionation of plasma on DEAE. Eight 25 ml lots of plasma were placed in siliconized vessels and heparin was added to four to a final concentration of 100 units/ml. After this point, operations were

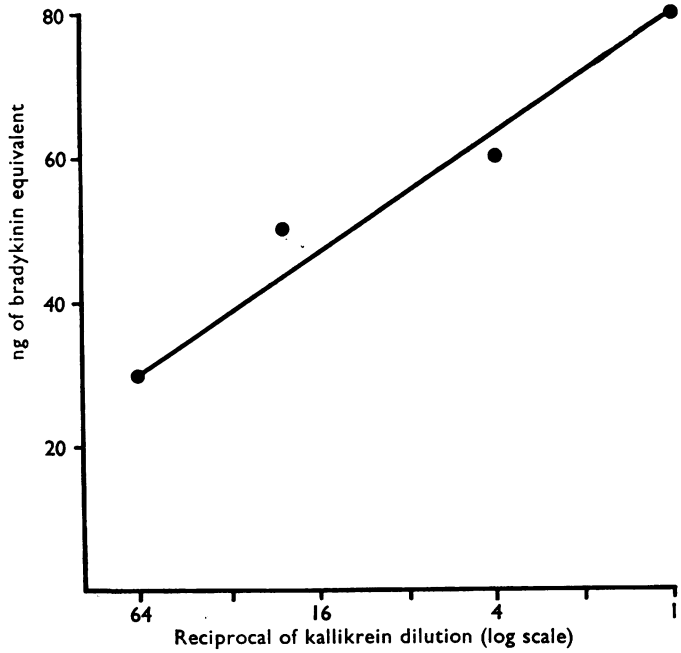

FIG. 2. Bradykinin equivalent released from standard substrate by graded dilutions of a kallikrein fraction after 36 min at 37° C, pH 7·5, ionic strength 0·15.

carried out in glass vessels. The eight samples were dialysed against 0·025 M Tris-HCl pH 7·6 and then each was adsorbed on to approximately 55 g of DEAE and allowed to stand for 1 h at +4° C before elution with 85 ml of 0·025 M Tris-HCl pH 7·6. The effluents were concentrated approximately 10-fold by ultrafiltration and assayed for prekallikrein and kallikrein. For this preliminary work the material retained on the DEAE was not analysed.

Table 1 shows the increase obtained in the ratio of prekallikrein to kallikrein, when heparin was added to the plasma before fractionation. From these results it appears that heparin retards the activation of prekallikrein. It should be noted that the total of prekallikrein and kallikrein obtained fluctuated from one experiment to another, but, in general, heparin appeared to decrease this amount.

Isolation of prekallikrein

DEAE batch procedure

Plasma containing heparin 100 units/ml (500 ml) was applied to 2·5 kg of DEAE-52 and eluted in a stepwise fashion. The first protein peak was collected in one fraction of about 1,000 ml. The remainder of the effluent was collected in 500 ml fractions. All fractions were examined for total protein and heparin content and for the ability to release kinins and to increase vascular permeability before and after activation with coated ballotini. All fractions devoid of kinin-releasing or permeability-increasing activities were concentrated at least tenfold and retested.

Figure 3 summarizes the results of a fractionation. A star above the fraction number indicates that the activity recorded was estimated from tests carried out on the fraction concentrated at least 10-fold ; the only exception is fraction 6 which, because of its high protein content, could be concentrated only 4-fold. Since some activity may have been lost during the concentration, the values for the concentrated fractions may not accurately represent the activity in the original dilute material, but are the best obtainable.

Fractions containing kallikrein. Fractions 1, 4 and 14 contained kallikrein. The ratios of prekallikrein to kallikrein were similar for fractions 1 and 4, being 1·1 : 1·0 and 0·9 : 1·0. Fraction 14 had no detectable prekallikrein and, in fact, its kallikrein activity decreased with exposure to coated ballotini. In fractions 1 and 14, the permeability-increasing activity changed in parallel with the kallikrein activity, but in fraction 4 it was unchanged by exposure to coated ballotini, instead of increasing as did the kallikrein activity. Of the total combined prekallikrein and kallikrein activity recovered, fraction 1 accounted for 51%, fraction 4 for 6% and fraction 14 for 43%. The permeability-increasing activity in these fractions accounted for only 18% of the total recovered, whereas that in the fractions without

TABLE 1. *Retardation of prekallikrein activation by heparin during DEAE fractionation of plasma*

Plasma sample number	Ratio of prekallikrein to kallikrein		Total prekallikrein plus kallikrein (doses)	
	Heparin added	No heparin	Heparin added	No heparin
1	52·1	3·2	526	4,040
2	30·7	1·3	1,510	4,460
3	34·6	1·7	719	4,060
4	143·0	1·0	4,890	18,500

kallikrein accounted for 82%. Fraction 1 contained the first protein peak eluted. Fraction 5 was associated with the first sharp rise in conductivity and elution of the second protein peak. Fraction 14 was associated with the second sharp rise in conductivity and the elution of heparin.

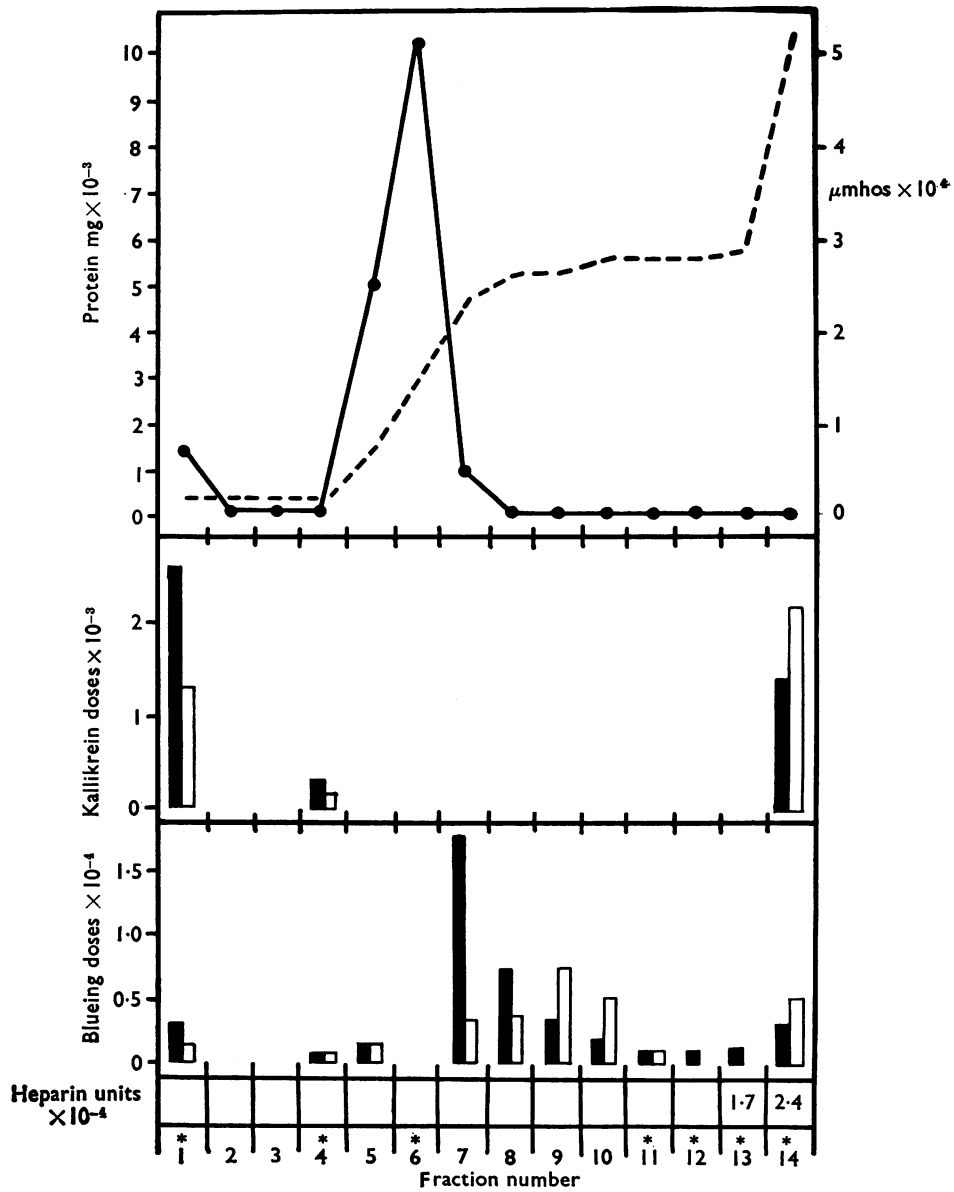

FIG. 3. DEAE fractionation of heparinized plasma. Plasma (525 ml) in the presence of heparin (100 units/ml) was dialysed against 0·025 M Tris-HCl pH 7·6 and then applied to 2·5 kg of DEAE-52 that had been equilibrated with the same buffer. Stepwise elution was carried out. Fraction 1 contained 945 ml and the remaining fractions about 500 ml each. The pH remained relatively constant between 7·6 and 8·0. The effluent was monitored for: total protein (——); conductivity (- - -); pre-activation kallikrein content and permeability activity (□); post-activation kallikrein content and permeability activity (■); heparin content. * Concentrated fractions (see text).

Fractions with permeability-increasing activity but no kallikrein. Though fractions 5 and 7–13 had permeability-increasing activity, they contained no kallikrein. This permeability activity without kallikrein was divisible into three main sectors. The first was fraction 5, its activity coming down before the major protein peak and remaining unchanged by exposure to coated ballotini. The activity appeared to be distinct from that in fractions 7–13 because none was demonstrable in fraction 6, even after fourfold concentration.

The second sector consisted of fractions 7 and 8. The activity came down after the major protein peak and increased after exposure to coated ballotini. Permeability-activity in the third sector, fractions 9 and 10, decreased after exposure to coated ballotini. Finally, there was trace activity in fractions 11, 12 and 13.

Distribution of heparin and protein. The precipitate formed when the heparinized plasma was dialysed against 0·025 M Tris-HCl pH 7·6 at +4° C, contained about 1% of the total heparin added, and when the dialysed heparinized plasma was fractionated on DEAE, heparin was demonstrable only in fractions 13 and 14 (Fig. 3). The amount was equivalent to 94% of the heparin applied to the DEAE. The protein content of fractions 5 and 6 was too high (greater than 2·0 mg/ml) for an accurate heparin assay. But because protein in high concentration causes falsely high readings for heparin, and the readings for these fractions were less than 1·6 units/ml, it can be assumed that they contained no significant amount of heparin.

The amount of protein in each fraction was calculated from the nitrogen content. Its recovery from the DEAE was 98·6% of the original applied.

Concentration and DFP treatment of prekallikrein

Fraction 1 was a dilute mixture of prekallikrein and kallikrein. The next step, concentration of the material and removal of kallikrein activity, was accomplished by ultrafiltration and treatment with 1×10^{-2}M DFP. The results with a typical batch are shown in Table 2. Preparation I is fraction 1 from DEAE ; preparation II is this material concentrated by ultrafiltration ; and preparation III, the DFP-treated, concentrated material. The total prekallikrein and kallikrein activity of preparation I was 3,140 kallikrein doses before ultrafiltration and 2,580 after—a loss of 18%. As DFP treatment necessarily involved a twofold dilution of the material, the concentration of prekallikrein in preparation III is half that in preparation II. The total prekallikrein content is the same in preparation III as that in preparation II, however, and the kallikrein activity has disappeared. The permeability increasing activity present in the different stages paralleled the kallikrein activity.

TABLE 2. *Prekallikrein and kallikrein content of fraction* 1 *after concentration and DFP treatment*

	Preparation I (dilute fraction 1)	Preparation II (concentrated fraction 1)	Preparation III (DFP treated fraction 1)
Kallikrein doses per ml	<3·3	22·6	<3·3
Prekallikrein doses per ml	<3·3	24·8	13·3
Total kallikrein doses	<3,140	1,230	<360
Total prekallikrein doses	<3,140	1,350	1,450
Total kallikrein plus prekallikrein doses	3,140	2,580	1,450
Blueing doses per ml before activation	<5·0	25·0	<5·0
Additional blueing doses per ml post activation	<5·0	28·0	10·5

Properties of prekallikrein

Behaviour on DEAE and immunoelectrophoresis

Neither isolated prekallikrein nor kallikrein was adsorbed to DEAE in the presence of 0·025 M Tris-HCl pH 7·6 and conductivity $0{\cdot}20 \times 10^4$ μmho. By immunoelectrophoresis with goat anti-human IgG and rabbit anti-whole human serum, the major contaminant of the isolated prekallikrein and kallikrein proved to be the immunoglobulin IgG.

Effects of DFP and SBTI

A kallikrein solution containing 106 kallikrein doses/ml was inhibited more than 32-fold when treated with 1×10^{-2} M DFP. The activability of prekallikrein is not affected by this concentration of DFP (Table 2). A solution of kallikrein (53 kallikrein doses/ml) prepared from isolated prekallikrein was inhibited more than 16-fold by SBTI 1·25 μg/ml.

Tests for activators in preparations of isolated prekallikrein

The following tests were made to determine whether there was a contaminating activator in the prekallikrein fraction, and whether prekallikrein could activate autocatalytically. First, a kallikrein solution too weak to release detectable kinin (1·66 kallikrein doses/ml) was incubated for periods up to 4 h with a prekallikrein solution (106 prekallikrein doses/ml) at 37° C, pH 7·5, ionic strength 0·15. There was no increase in kallikrein activity.

Second, when a solution of prekallikrein (14 prekallikrein doses/ml) was exposed to ballotini coated with kallikrein (14 kallikrein doses/ml) there was no increase in activity.

Test for the presence of inhibitor in isolated prekallikrein

The following test was made for an inhibitor of kallikrein in the prekallikrein fraction. Constant volumes of a prekallikrein solution (53 prekallikrein doses/ml pH 7·5, ionic strength 0·15) were added to equal volumes of serial 2-fold dilutions of a kallikrein solution containing 426 kallikrein doses/ml at the same pH and ionic strength. A control series was set up in which 0·01 M sodium phosphate in 0·15 M sodium chloride pH 7·5 was used in place of the prekallikrein solution. After 60 min at 37° C, the solutions were tested for their ability to release kinins. The prekallikrein solution did not diminish the kinin releasing activity of kallikrein.

Stability of prekallikrein

The prekallikrein fractions were stable for periods up to 6 h at room temperature, 48 h at +4° C and 8 months at −30° C.

Discussion

The isolated prekallikrein was stable in solution at low temperature and was free of activators, inhibitors or any contaminating kallikrein detectable by our assay method. It did not adsorb to DEAE in the presence of buffers with pH 7·6 and

conductivity, $0{\cdot}20 \times 10^4$ μmho, and its ability to be activated was not affected by DFP. The kallikrein formed from it released kinins, increased vascular permeability, was inhibited by DFP and SBTI, and did not adsorb to DEAE in the presence of buffers with pH 7·6 and conductivity to $0{\cdot}20 \times 10^4$ μmho. Whereas the prekallikrein was functionally pure, it was far from physico-chemically pure, the major contaminant being the immunoglobulin IgG.

Although the isolation of human prekallikrein has not previously been reported, human plasma kallikrein has been isolated by a number of workers. Probably the most highly purified preparation was obtained by the combined use of cold aqueous ethanol fractionation, DEAE and CM cellulose chromatography, and Sephadex G-200 gel filtration (Colman *et al.*, 1969). Three kallikreins, designated I, II and III, were isolated. Kallikreins I and II were closely related—kallikrein II possibly being a complex of kallikrein I with itself or some other protein. Kallikrein I was a slow γ-globulin with a molecular weight of 99,800 ; kallikrein II, a fast γ-globulin with a molecular weight of 163,000. Both were poorly adsorbed to DEAE and were arginine esterases susceptible to DFP, SBTI and plasma inhibitors of kallikrein. Kallikrein III, although an arginine esterase susceptible to DFP, migrated as an α-globulin and was not susceptible to plasma inhibitors or SBTI. It seems most likely that the isolated prekallikrein described in our paper is the precursor of the SBTI susceptible kallikreins I and II.

Fresh frozen plasma collected in plastic bags was used for the isolation. Although this material was adequate for our purposes, blood collected directly into siliconized vessels and processed immediately would probably have yielded more prekallikrein. No attempt was made to compare the amount of prekallikrein and kallikrein in whole plasma with that in the effluent from DEAE because the known presence of inhibitor activity in plasma renders any estimate of " plasma kallikrein concentration " difficult to interpret (Becker & Kagen, 1964).

In the DEAE fractionation, it is noteworthy that all the detectable prekallikrein comes down in fractions 1 and 4. In both, the ratio of prekallikrein to kallikrein activity is similar. The activity in fraction 4 probably represents the trailing portion of the peak of activity in fraction 1, eluted sharply by the sudden rise in salt concentration (Sober, Hartley, Carrol & Peterson, 1965). Fraction 14 contained only kallikrein. Its presence here is probably due to its binding to heparin, which does not elute until late in the fractionation procedure. The ability of heparin to bind to certain plasma proteins is well known (Engleberg, 1963), and this would explain the lowered yields of total kallikrein obtained in the initial eluate from fractionations of plasma containing heparin (Table 1). The alternative possibility, that heparin, in the concentration used, inhibits some of the plasma kallikrein, is unlikely in the light of the work of McConnell, Kagen & Becker (1965).

It is of interest that no prekallikrein was recovered in fraction 14. If prekallikrein as such is bound to heparin, it must have been fully activated by the time it was eluted from the DEAE, in spite of the high concentration of heparin. Alternatively, the high heparin concentration may have inhibited the activation of any prekallikrein present.

The decrease in kallikrein activity in fraction 14 after exposure to coated ballotini (Fig. 3) is not surprising ; Colman *et al.* (1969) also noted a decrease in the activity of kallikrein after exposure to foreign surfaces. It is probably due to adsorption of kallikrein to the surface.

The mechanism by which heparin retards the activation of prekallikrein during fractionation is not clear. At least three explanations can be offered. First, an activator may be precipitated by heparin during the initial dialysis. Heparin is known to precipitate certain proteins at +4° C (Smith & Von Korff, 1957) and it was present in the precipitate that formed in the plasma during dialysis at +4° C; second, it may interfere with an activator by forming a complex with it; and third, it may complex with prekallikrein and prevent its activation.

The permeability-increasing activity in fractions 1 and 14 paralleled the kallikrein activity and most likely can be attributed directly to it. That it did not do so in fraction 4 is probably due to contamination of the kallikrein with the permeability factor of fraction 5.

Most of the permeability-increasing material recovered lacked kallikrein activity. It emerged from the DEAE in three main elution sectors. The evidence that the activity in each of these sectors was due to separate substances is derived from the differing behaviour of the activity in the fractions on exposure to coated ballotini. Exact identification of the responsible substances must await more precise methods of isolation and characterization.

Tentatively, the activity in fractions 7 and 8 can be identified as due to Pf/dil, because it increases after exposure to coated ballotini. That in fractions 9 and 10 may be due to Hageman factor, for it decreases after exposure to coated ballotini, and Hageman factor might well adsorb to the ballotini (Ratnoff & Miles, 1964). The substances to which the activity in fraction 5 and fractions 11, 12 and 13 is due cannot be identified even tentatively, but one of them may be plasmin (Ratnoff, 1965).

This tentative identification of the permeability-increasing substances without kallikrein activity accords, in general, with the identification by Webster & Pierce (1966) of similar substances isolated by DEAE chromatography in whole plasma.

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