Effect of Heparin on the Kinin-Forming Activity of Trypsin, Plasmin, and Various Kallikreins[1] (34555)

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Heparin has been shown to inhibit such proteases as trypsin (1–3), pepsin (4), thrombin (5), human plasma kallikrein and Pf/dil[2] (6). The latter two enzymes were able to be distinguished from each other by the relative degree of inhibition by heparin. The release of heparin in experimental shock states associated with protease activation (7) and the use of heparin as an anticoagulant in studies involving the assay of kinin-forming enzymes, provoked this present investigation. The effect of heparin on the *in vitro* kinin-forming activity of the following proteases was studied: crystalline trypsin, streptokinase-activated human plasmin, human plasma kallikrein, dog plasma kallikrein, and dog salivary kallikrein.

Materials and Methods. Crystalline bovine trypsin was obtained from Worthington Laboratories, Freehold, New Jersey. Streptokinase-activated human plasmin was a product of Parke Davis and Co. (Fibrinolysin) with an activity of approximately 10 RPMI[3] plasmin units/mg protein. Human and dog plasma kallikrein were prepared by precipitation with 10 times the volume of 20 % acetone according to a previously published method (8). The precipitated plasma solution was permitted to stand overnight at 4°, centrifuged at 2500 rpm, the precipitate collected and lyophilized. Dog salivary kallikrein was prepared from parotid saliva collected by direct duct cannulation technique. The saliva was filtered through a Whatman No. 1 filter paper, treated with three times the volume of 20 % acetone, permitted to stand overnight at 4°, centrifuged at 3500 rpm for 30 min, and lyophilized.

Heparin was avaliable from Abbott Laboratories (Pan-Heparin) at a concentration of 50,000 units/ml. Other inhibitors used included Trasylol (product of Farbenfabriken Bayer AG, Germany), soya bean trypsin inhibitor, and ovomucoid obtained from Worthington Laboratories, Freehold, New Jersey.

Human kininogen substrate was prepared from ACD plasma heated to 80° and placed immediately on ice. The precipitated protein was homogenized in a Waring Blendor, centrifuged at 3500 rpm, and the supernatant collected. Approximately 50 ml of plasma made 20 ml of heated plasma substrate which contained 35 % of the kinin-yielding activity of the original plasma. The kininogen substrate was stable when stored frozen in a 0.6 % protein concentration solution. The substrate did not have any kinin-forming or kinin-destroying activity. Pf/dil was unable to form kinin from the heated substrate (6). Incubation of 0.005 μg trypsin with 1.2 mg of substrate yielded 0.14 μg of kinin.

The kinin-forming activity generated by acetone treatment of heparinized or citrated dog and human plasma first was studied. Plasma was placed into 10 times the volume of 20 % acetone, and the precipitate, collected after overnight incubation at 4°, was reconstituted to the original volume of plasma with 0.1 *M* Tris buffer, pH 7.8. Increasing volumes of reconstituted precipitate were incubated with 1.2 mg of kininogen substrate for 30 min at 37°. Kinin formed was estimated on the isolated rat uterus perfused at room temperature with modified Tyrode's solution containing a reduced calcium concen-

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[2] Permeability factor.

[3] Roswell Park Memorial Institute.

TABLE I. Kinin-forming capacity of varying volumes of citrated human plasma, citrated mature dog plasma, and heparinized puppy and dog plasma.
(Plasma samples were incubated with kininogen substrate.)

Plasma source	Kinin formation with varying plasma volumes (ml)					
	0.025	0.05	0.1	0.25	0.5	1.0
ACD human	—	+	++++	++++	++++	0
ACD carcinoid	—	0	++++	++++	++++	0
Heparinized puppy	—	—	—	—	—	—
Heparinized mature dog	—	—	—	—	—	—
Citrated mature dog	++	++++	++++	++++	++++	++++

Note: — = No kinin formation.
+ = Partial kinin formation.
++++ = Total kinin formation.
0 = Not tested.

tration (9). Synthetic bradykinin (Sandoz), 8.3×10^{-9} g/ml, was used as a reference standard.

The effect of heparin on the kinin-forming activity of trypsin, plasmin, and kallikreins was studied by incubating 0.1 ml of kininogen substrate (1.2 mg), and 0.1 ml of the enzyme. The concentration of enzymes used was predetermined on the basis of the maximum kinin released from the substrate by the minimum amount of enzyme. Thus, the enzyme activities were comparable. All the samples were made up to a final volume of 1.0 ml by the addition of 0.1 *M* Tris buffer, pH 7.8. After 30 min of incubation at 37°, the reaction was stopped by the addition of 1.0 mg of soya bean trypsin inhibitor. Kinin was assayed on the isolated rat uterus. The effect of Trasylol, ovomucoid, soya bean trypsin, and lima bean trypsin inhibitor on dog plasma kallikrein was compared to that of heparin.

Results. Kinin-forming activity of various acetone-treated plasmas. Table I summarizes the results obtained with varying volumes of ACD human plasma, citrated plasma from a patient with carcinoid, heparinized plasma from a month-old puppy, heparinized plasma from a mature dog, and citrated plasma from a mature dog. Only the heparinized plasma samples did not form kinin following incubation with kininogen substrate. 1, 10-phenanthroline was added to destroy kininase. The amount of heparin in the dog plasma samples was estimated at 100 μg per ml. The citrated dog plasma samples formed kinin at volumes as low as 0.025 ml.

Effect of heparin on human and dog plasma. Heparin in equivalent doses inhibited more strongly the kinin-forming activity of dog plasma compared to human plasma. Table II summarizes the results. A heparin concentration of 2.5 units completely inhibited the kinin-forming activity of 0.1 ml of dog

TABLE II. Effect of heparin on kinin-forming capacity of mature dog plasma and human plasma.

Plasma source	Kinin formation with varying concentrations of heparin (units/ml)					
	1.0	2.5	5.0	20	200	2000
Mature dog (0.1 ml)	++++	—	—	—	—	—
Human (0.1 ml)	++++	++++	++++	+++	++	+

Note: — = No kinin formation.
+ = Partial kinin formation.
++ = 50% kinin formation.
+++ = More than 50% kinin formation.
++++ = Total kinin formation.

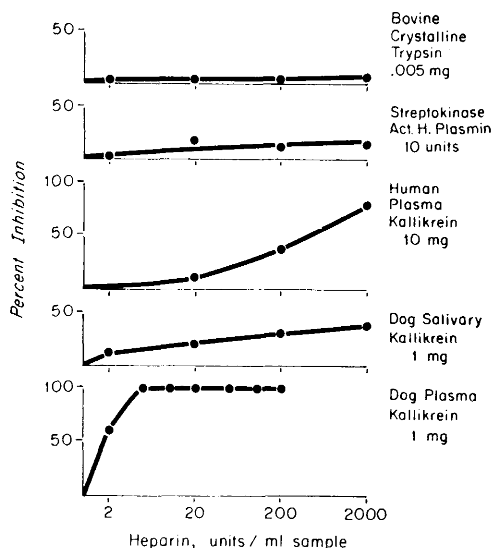

FIG. 1. Effect of varying concentrations of heparin on the kinin-forming activity of bovine trypsin, human plasmin, human plasma kallikrein, dog salivary kallikrein, and dog plasma kallikrein.

plasma, whereas 2000 units of heparin were not quite sufficient to abolish the kinin-forming activity of human plasma.

Effect of heparin on kinin-forming activity of various proteases. Figure 1 presents graphically the inhibitory effect of heparin on the kinin-forming activity of bovine trypsin, human plasmin, human plasma kallikrein, dog plasma kallikrein, and dog salivary kallikrein. It will be noted that 2000 units of heparin did not inhibit to any significant extent the kinin-forming activity of 5 μg of crystalline trypsin. Human plasmin was inhibited only 13–18 % by the highest concentration of heparin. Human plasma kallikrein, however, was inhibited 80 % by the high heparin concentration (2000 units) and 35 % inhibited by 200 units of heparin. Dog salivary kallikrein was inhibited approximately 40 % by the highest concentration of heparin studied. In sharp contrast to the above data, 5 units of heparin inhibited dog plasma kallikrein 100 %, while as little as 2 heparin units resulted in almost 60% inhibition. These data correlate with those seen in Table I.

Effect of protease inhibitors on kinin-forming activity of dog plasma kallikrein. The remarkable inhibitory action of heparin on dog plasma kallikrein was equivalent to 0.1 mg of soya bean trypsin inhibitor and 500 units of Trasylol, as seen in Table III. One-mg concentrations of lima bean trypsin inhibitor caused partial inhibition, while 1 mg of ovomucoid had no inhibitory action.

Discussion. By virtue of its strong electronegative charge, heparin is capable of binding to and interfering with the action of many proteins, including enzymes (10). Remarkable inhibition of renin was achieved with heparin concentrations as low as 5 units/ml (11). The heparin was highly purified and was devoid of antitryptic activity. In this instance, inhibition was based on a reversible competition with renin substrate for the active enzyme site. Caution was advised relative to interpretation of renin-angiotensin data obtained from heparinized blood samples.

The data in the present study demonstrate the extraordinary ability of heparin to inhibit the kinin-forming activity of dog plasma kallikrein. The dog is used in many experimental shock models involving assay of kinin formation in the presence of exogenous or

TABLE III. Comparative effect of heparin and protease inhibitors Trasylol, lima bean trypsin inhibitor (LBTI), soya bean trypsin inhibitor (SBTI), and ovomucoid (OM) on the kinin-forming activity of dog plasma kallikrein.

	Kinin formation with heparin and protease inhibitors				
Protease	Heparin 5 units	SBTI 0.1 mg	LBTI 1 mg	OM 1 mg	Trasylol 500 units
Dog plasma kallikrein	—	—	++	++++	—

Note: — = No kinin formation.
++++ = Total kinin formation.
++ = Partial kinin formation.

endogenously-liberated heparin. A similar note of caution relative to interpretation of data so obtained would appear appropriate. The inhibition appears rather specific to a particular kallikrein species. Dog salivary kallikrein was inhibited only limitedly while human plasma kallikrein was inhibited 80 % at only very high doses of heparin. An almost identical range of inhibition by heparin of the permeability activity of partially purified human plasma kallikrein was reported by McConnel *et al.* (6), who recorded 50 % inhibition at a heparin concentration of 1950 units/ml.

Heparin did not interfere with the kinin-forming activity of either trypsin or plasmin. Both enzymes are capable of forming kinin from suitable substrate (9, 12). Heparin has been reported both to enhance (13–15) and inhibit (16, 17) *in vitro* fibrinolytic activity of plasmin, while von Kaulla (18) found fibrinolysis inhibited by high concentrations of heparin and enhanced by low concentrations. Recently heparin was reported to act as an inhibitor of plasminogen activation in the same manner as epsilon aminocaproic acid (19). The studies of Astrup *et al.* (20) reveal commercial preparations to be contaminated with protease inhibitors. Thus, purified heparin preparations were not able to inhibit the effect of both trypsin and plasmin on heated and unheated bovine fibrin as well as casein. Trypsin-inhibitory fractions were separated from the heparin preparations by column chromatography. These contaminants were of an acid nature differing from both the soya bean trypsin inhibitor and trypsin inhibitor (mingin) from human pregnancy urine. In previous studies, a wide range of natural and plant inhibitors were found to inhibit the kinin-forming activity of trypsin and plasmin (9). Studies with different preparations of heparin are required to clarify this question further.

Conclusion. The effect of heparin on the kinin-forming activity of trypsin, plasmin, and kallikrein from human plasma, dog salivary gland, and dog plasma was studied. Heparin at concentrations of 5 units/ml was a potent inhibitor of dog plasma kallikrein. This inhibitory activity was equivalent to 500 units of Trasylol or 0.1 mg soya bean trypsin inhibitor. At high concentrations (2000 units/ml), heparin inhibited 80 % of human plasma kallikrein activity and 40 % of dog salivary kallikrein activity. The kinin-forming activity of neither trypsin nor human plasmin was inhibited.

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