Extraction of Plasminogen Activator from Canine Vascular Segments *in Situ*[1] (34317)

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The local release of plasminogen activator into blood in vascular segments which are occluded from the general circulation occurs *in vivo* (man) during the ischemic state (1, 2), after paravenous injection of vasoactive drugs (3) or following various stimuli inducing fibrinolytic activity in the general circulation (4). *In vitro*, the release of plasminogen activator occurs from the endothelium of everted veins (5, 6), from venous endothelial strips (7), and from microsections of veins (8). Nonfibrinolytic blood infused into emptied varicose veins acquires fibrinolytic activity (9). This report describes attempts at extracting plasminogen activator from vascular segments *in situ* with fluids other than blood and attempts to identify the best conditions under which this extraction can be achieved.

Material and Methods. Animals. Healthy dogs of various breeds and both sexes weighing 15–40 kg were used.

Chemicals and reagents. Sodium pentobarbital 65 mg/ml (Napental Massengil Comp.), normal sodium chloride (Travenol), buffered saline (4 parts barbital acetate buffer, pH 7.4 [10], one part normal sodium chloride), Ringer solution, absolute ethanol (U.S. Industrial Chemicals Comp.), and ouabain (Lilly), all dissolved in Ringer solution; digitonin (Nutritional Biochemicals Corp.) was dissolved in a small amount of absolute ethanol and the Ringer solution was added. The resulting suspension was thoroughly mixed before each application. Final concentrations: digitonin 2×10^{-4} *M*, alcohol 1.25% (by volume), ouabain 6.86×10^{-5} *M*.

Surgical preparation of vessels. Anesthesia was induced with 30–35 mg/kg of iv sodium pentobarbital and maintained with additional 1–2 ml of pentobarbital as required. A 5–6-cm segment of vessel was isolated by blunt dissection and freed from all adjacent structures with minimal trauma. Tributary branches were ligated with 3-o surgical silk (Ethicon, Inc.) and divided.

Femoral vein: The vein was ligated distally and a large tributary branch near the ligation catheterized with a 5–15-cm length of Vivosil medical grade silicone elastic tubing No. 7002-040. The vascular segment was rinsed repeatedly with 2 ml of NaCl through the tubing until the effluent was free of blood and then clamped proximally with a small hemostat. Femoral artery: The same procedure was performed except that the artery was clamped proximally first. External jugular vein: One of the two large tributaries was cannulated with the silicone tubing and the other was clamped. The cannulated vein was rinsed free of blood with saline and then clamped off distally. To avoid drying, all vessels were packed in saline soaked gauze and wrapped in parafilm.

Extraction of plasminogen activator: The solutions to be tested were injected slowly through the catheter into the empty vessel, avoiding distention carefully, and retained for various lengths of time and under various conditions. The extracting fluid was withdrawn, its pH was readjusted to 7.42 where indicated and centrifuged at 25,000*g*. for 15 min at 4°.

Estimation of plasminogen activator activity: Fibrin plates either made up with bovine fibrinogen (Armour) (11) or in most cases with plasminogen enriched fibrinogen (12) were used. A 0.03-ml sample of the centrifuged extraction fluid was applied in triplicate on the plates, which were incubated at

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TABLE I. Effect of Extraction Time, Ion Strength, and Temperature on the Release of Plasminogen Activator from Ligated Segments of Canine External Jugular Veins *in Situ*.[a]

	A Extraction time (min)			B Ion strength			C pH		D Temp (°C)		
					NaCl (%)						
	15	30	45	H_2O	0.85	1.7	5	7.4	4	23	37
Av lysed area	130	150	309	273	229	186	172	104	8	39	39
Veins used	10	7	5	2	2	2	2	2	3	3	3

[a] The extraction time, except where otherwise indicated, was 30 min at room temperature. Buffered saline pH 7.4 and 5, respectively, was used except for ion strength expt. Plasminogen enriched fibrin plates were used except for bovine fibrinogen (Armour) in the temperature experiments. For further explanation see text.

37° for 18 hr. The average value (mm^2) of the three lysed areas was used for tabulation and graphic display. The difference between the three measurements was less than 10%.

Results. General remarks: Without being distended, the segments of the external jugular vein could hold between 1.5–2 ml of the test fluid while the femoral veins and arteries held approximately 0.5–1.0 ml.

Effect of extraction time on the amount of extracted activator activity: The venous segment was filled with buffered saline and aliquots were withdrawn at intervals. The results are shown in Table IA. On their basis, a retention time of 30 min was adapted as standard procedure. In some individual experiments, a drop was observed when the retention time exceeded 15 min.

Release of plasminogen activator from the various vessels: The results of these experiments are given in Table II. The activator activity of the contralateral vein varied considerably. The external jugular vein is a far better source of activator than either the femoral vein or artery.

Stability of extracted plasminogen activator: Extracts from external jugular veins of two dogs produced after various preincubation times (37°) the following average lysis zones (mm^2): 0 hr, 159; 1 hr, 110; 2 hr, 104; 3 hr, 110; 5 hr, 83. Therefore it appeared that after some initial loss, the activator activity became rather stable for at least 3 hr permitting meaningful experiments during that period.

Temperature and plasminogen activator release: This correlation was studied by the additional ligation of the isolated segment of the external jugular veins in their midportion forming two separated segments of the same

TABLE II. Comparison of Plasminogen Activator Activity Extractable from Segments of Canine Blood Vessels *in Situ*.[a]

Blood vessels	Lysed area (mm^2)								Av	Ratio[b]
Jugular vein, left	348	120	435	272	190	145	70	100	211	205	16/0
right	258	209	480	163	127	164	49	144	200		
Femoral vein, left	202	112	106	144	0	64	181	81	111	80	13/2
right	52	25	16	192	30	—	0	25	50		
artery, left	—	27	0	81	0	0	9	36	22	18	6/8
right	—	0	0	72	0	0	0	25	14		
Dog no.	1	2	3	4	5	6	7	8			

[a] Extraction time: 30 min at room temperature; extraction medium: barbital acetate buffered saline, pH 7.42; activity expressed as lysed areas (mm^2) on plasminogen-enriched unheated bovine fibrin plates; p values: jugular v. vs. femoral v. <0.05; jugular v. vs. femoral artery <0.02; femoral v. vs. femoral ar. <0.01.

[b] Ratio of vessels yielding activity/vessels yielding no activity.

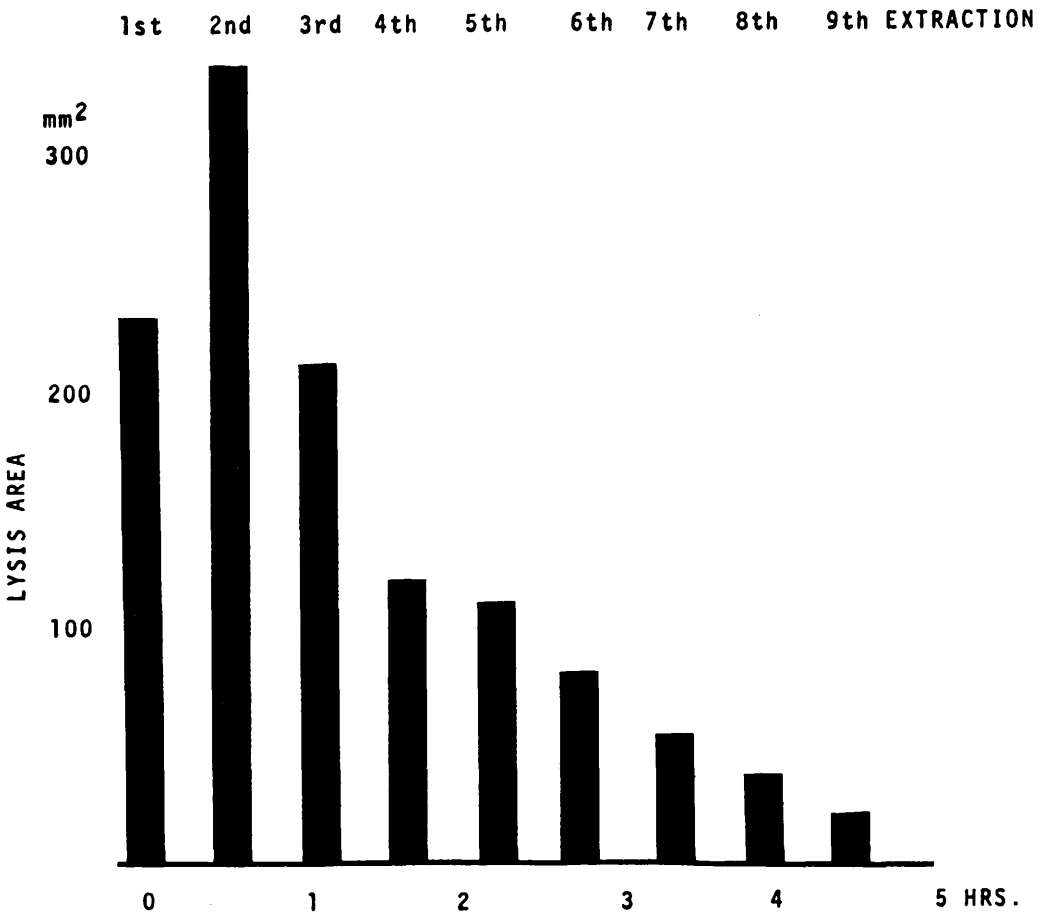

FIG. 1. Serial extractions of plasminogen activator with Ringer solution from an isolated section of canine external jugular vein *in situ*; lysis area (mm^2) produced by extracts on unheated bovine fibrin plates. Note the gradual exhaustion of plasminogen activator.

vein. Buffered saline at two different temperatures (4 and 23°; 4 and 37°; 23 and 37°) was retained for 30 min within the twin segments and then tested (Table ID). For the following 30 min, the placement of the new buffered saline solutions was reversed and the results again were compared. Vascular segments which had not released plasminogen activator at 4°, subsequently released a normal amount at 37°.

pH and plasminogen activator release: This correlation was also studied with the two-segment technique using saline solutions, buffered at pH 5 and 7.4 (Table IC). After apparent exhaustion of the vessel with repeated extractions at pH 7.4, plasminogen activator release could again be elicited with the acid saline solution.

Ion concentration and plasminogen activator release: With the two-segment technique, the efficiency of plasminogen extraction by distilled water, 0.85 and 1.7% NaCl were compared (results in Table IB).

Effect of serial extraction on plasminogen activator release: Seven to 9 serial 30-min extractions with Ringer solution at room temperature were performed. Of the 17 jugular vein segments, one was exhausted after 3, and three veins after 6 extractions. Thirteen veins were still releasing some activator after 7–9 extractions and a total experiment duration of 4–5 hr. A typical run for an external

jugular vein is shown in Fig. 1. Of the 17 femoral veins, three were exhausted after 1, four after 3, and seven after 4–7 extractions. After these serial extractions, histologic study of the jugular veins revealed an essentially normal endothelial lining.

Enhancing effect of digitonin on plasminogen activator release: Ouabain and digitonin were added to the extraction fluid. An ethanol control was run because the Ringer solution with digitonin contained 1.25% ethanol, required for its solubilization. The solutions containing compounds were retained for 30 min in one venous segment while Ringer solution alone filled the contralateral vein. Each dog thus served as its own control. Six dogs were tested on ouabain, 2 on ethanol and 17 on digitonin. The results are shown in Fig. 2. Digitonin very markedly enhanced plasminogen activator release from both the jugular ($p < 0.05$) and femoral veins ($p < 0.1$). The release also appeared to be more sustained. In 25 experiments, one venous segment was subjected to eight extractions with Ringer solution plus digitonin and the contralateral segment to the same number with Ringer solution alone. With digitonin, only 7 of 25 veins became exhausted as compared to 16 of 25 with Ringer solution.

Attempts at renewed extraction after 24 hr: After the extractions with buffered saline, the ligatures were removed from six veins in three dogs to restore circulation. The skin was closed and 1,000,000 units of penicillin was given im. Twenty-four hr later, upon reexamination, the walls of the veins were thickened and four of the six veins contained thrombi which were extricated. Ringer solution was unable to extract plasminogen activator activity from any of these veins. However, some activity could be extracted in the presence of digitonin.

Heated plates: No lysis areas were ever obtained on heated plates (buffered saline extracts from 16 jugular and 16 femoral veins of eight dogs).

Discussion. The primary finding of this investigation is the surprisingly easy recovery of plasminogen activator from venous walls (left *in situ*) in the dog by fluid placed in

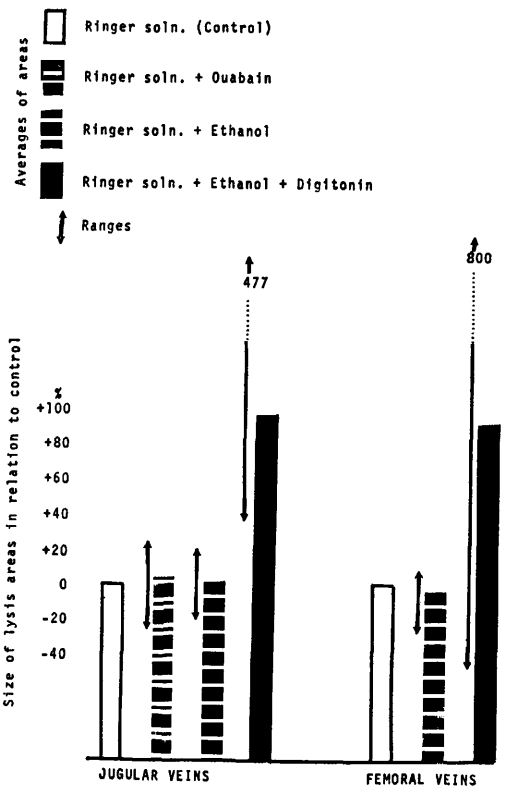

FIG. 2. Enhancement of plasminogen activator release from isolated segments of canine venous walls *in situ*; Ringer solution alone: 100%. Note enhancement of plasminogen activator release by addition of digitonin 0.0002 *M* to the extraction fluid.

the vascular lumen. This release, although gradually decreasing with time may go on for hours as purposeful attempts to exhaust the releasing mechanism have indicated. The recovery is enhanced by acidity and blocked by low temperature of 4°. The higher efficiency of water as an extracting fluid could be explained by osmotic damage to the cell membrane. A similar explanation may also hold true for the markedly enhanced effect on extraction produced by digitonin which has been shown to induce damage (possibly perforation) to the endothelial membrane as observed in Rous sarcoma virus and erythrocytes (13). This would permit a more extensive diffusion of plasminogen activator from the endothelium itself. In cell cultures diffusion (or extraction?) of plasminogen activator into the ambient fluid has been observed

and is the actual basis for the fibrin slide technique. The origin of the vascular plasminogen activator primarily appears to be the microsomal fraction of the endothelium as was shown in the bovine jugular vein (14). If this is so, one would actually be dealing with tissue activator which is known to be stable, and varying degrees of stability resulting from the different extraction procedures would not enter into the results reported here. The present study also confirms the observations that veins exhibit more plasminogen activator activity than arteries (6, 15).

Summary. Plasminogen activator is easily recovered from canine veins *in situ* by solutions retained in vessel lumens, previously emptied of blood. The veins can be extracted repeatedly before they become exhausted. A pH of 5, low ion strength, and the presence of digitonon will enhance the plasminogen activator release. Low temperatures will block it. The external jugular vein generally yielded more plasminogen than did the femoral vein. The femoral artery yielded very little activator.

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