THE EFFECT OF INTRAVENOUS INFUSION OF THROMBOPLASTIN ON "HEPARIN TOLERANCE" [1]

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The widespread use of anticoagulants in the prophylaxis and therapy of thrombo-embolic disease has stimulated interest in methods for selecting patients who are predisposed to intravascular clotting. It has been suggested but by no means proved that inherent hypercoagulability of the blood is a frequent factor in the pathogenesis of thrombosis. On the basis of this belief several clinical tests have been devised to detect hypercoagulability of the blood. Increased resistance to the anticoagulant activity of heparin has been reported to occur in patients with thrombotic disease. The clotting time of blood drawn following intravenous injection of heparin was said to be shorter in patients predisposed to thrombosis than in normal individuals receiving the same amount of heparin (1, 2). Similar results were obtained with blood heparinized *in vitro* (3). Increased resistance to heparin has been attributed to the liberation of thromboplastin into the blood following surgical operations and in certain other conditions in which thromboses frequently occur (3–5).

Thromboplastic substances accelerate the coagulation of blood and can overcome the anticoagulant effect of heparin *in vitro* (6). However, no evidence is available to support the belief that thromboplastin liberated into the circulating blood can cause an increased resistance to the anticoagulant action of heparin. The effect of the intravenous injection of thromboplastin on "heparin tolerance" was therefore studied. Experiments were performed in which dogs were given intravenous infusions of homologous brain thromboplastin. "Heparin tolerance" was measured repeatedly before, during, and after the infusions. In no instance was increased resistance to heparin noted. Administration of small amounts of thromboplastin resulted in exaggerated response to heparin, and larger amounts delayed the coagulation of blood.

METHODS AND MATERIALS

Healthy mongrel dogs weighing 7.3 to 13.6 kilograms were anesthetized by the intravenous injection of sodium pentobarbital, and anesthesia was maintained for three to six and one-half hours by repeated injections of the drug as necessary. A thromboplastin preparation was infused from a calibrated buret through a 20 gauge needle into a saphenous vein. Blood was drawn from the jugular veins using 20 gauge needles and silicone-treated syringes. At intervals before, during, and after the infusion of thromboplastin, specimens were obtained for clotting studies.

Preparation of thromboplastin. Thromboplastin was prepared from acetone-extracted dog brain by the method of Brambel (7). The resulting suspension was centrifuged in a Servall angle centrifuge at 11,500 r.p.m. for 10 minutes to remove particulate material. The slightly opalescent supernatant fluid contained from 6 to 12 mg. of brain solids per ml. as measured by drying *in vacuo*. The thromboplastic activity of this material was determined by testing its clot-accelerating effect on the plasma of the dog to be infused with the preparation. One-tenth ml. of thromboplastin was added to 0.1 ml. of oxalated plasma. This mixture was clotted at 37° C. by the addition of 0.1 ml. of M/40 calcium chloride solution. Various thromboplastin preparations clotted dog plasma in eight to 13 seconds. For intravenous use the thromboplastin was appropriately diluted with 0.85% saline solution and filtered through glass wool into the infusion apparatus. The thromboplastic activity of the diluted and filtered material was then determined as described above.

Clotting times. Clotting times were determined at 37° C., employing 1 ml. portions of blood in acid-washed 13 × 100 mm. Pyrex test tubes. Two tubes were used for each determination. The clotting time of the blood of normal dogs varied between 10 and 20 minutes, values essentially identical with those obtained in human subjects with the same technique.

In vitro "heparin tolerance." One ml. portions of freshly drawn blood were placed in Pyrex clotting tubes

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containing 0.1 ml. of heparin[3] solution. The final concentration of heparin was varied from 0.05 to 0.2 Toronto units per ml. of blood. The clot-retarding effect of the various heparin concentrations was determined by comparing the clotting time of heparinized and unheparinized blood. Each test was carried out in duplicate at 37° C. At least two control "heparin tolerance" tests were performed on each animal.

In vivo "heparin tolerance." After several control clotting times were performed, a measured volume of heparin solution was injected into a saphenous vein. The clotting time of blood drawn from the jugular vein was determined at intervals following the injection.

Prothrombin times. To freshly drawn blood was added 10% by volume of M/10 sodium oxalate solution. The prothrombin content of oxalated plasma was estimated by the one-stage method employing rabbit brain thromboplastin prepared by the technique of Brambel (7). In addition the prothrombin time of plasma diluted to 12½% was determined. The diluent employed was 0.85% sodium chloride solution or a solution containing six parts of saline and one part of M/10 sodium oxalate solution. In some experiments in which saline alone was used, diluted plasma clotted prior to the addition of calcium chloride solution. Prothrombin times on 12½% plasma were performed at least in triplicate.

Fibrinogen determinations. The fibrinogen concentration of oxalated plasma was determined by the method of Ratnoff and Menzie (8).

Platelet counts. Platelet counts were performed in duplicate by a direct method employing Rees-Ecker diluent.

Thrombin clotting times. The time necessary for clot formation after the addition of 0.2 ml. of a thrombin[4] solution to an equal volume of oxalated plasma was determined in duplicate at 37° C. Powdered thrombin was dissolved in barbital buffer to a concentration of 2.0 units per ml.

Barbital buffer. Barbital buffer was prepared from the following substances: sodium diethylbarbiturate 2.06 grams, diethylbarbituric acid 2.76 grams, sodium chloride 7.30 grams, and sufficient triple-distilled water to bring the total volume to one liter. To facilitate solution the mixture was heated gently without boiling. The pH of this solution was 7.5 ± 0.1.

EXPERIMENTAL STUDY

Normal dogs, anesthetized with nembutal, were given intravenous infusions of homologous brain thromboplastin. The thromboplastic activity of the preparations and the rate of infusion were varied over wide ranges. Animals receiving these infusions showed no evidence of untoward reactions except in those instances in which rapid injections of very potent thromboplastin were given. Pulse and respirations did not appear to be influenced by the infusions. No convulsive movements were noted. In no instance was there detectable thrombosis of the saphenous vein at the site of the thromboplastin infusion. Recovery from anesthesia was uncomplicated, and the animals appeared normal for a period of observation of at least one week. No animals were sacrificed.

When an injection of 10 ml. of a potent thromboplastin[5] was given within 30 seconds, the animal appeared to be in respiratory distress for about 15 minutes. Thereafter, recovery was prompt and uncomplicated. The administration of 10 ml. of even more potent thromboplastin (prothrombin time 13 seconds or less) within 30 seconds was followed by evidence of severe respiratory distress and death within two or three minutes. At autopsy large clots were found in the chambers of the right side of the heart, pulmonary arteries, and frequently in the inferior vena cava.

In vitro "heparin tolerance." Figure 1 gives the results of a typical experiment. This experiment will be described in detail:

A healthy 10 kilogram male mongrel dog was anesthetized by the intravenous injection of sodium pentobarbital, 30 mg. per kilogram. Anesthesia was maintained for about three hours by two subsequent injections of 25 mg. of the drug. Seventeen ml. of blood were obtained by jugular vein puncture with a 20 gauge needle and a 20 ml. silicone-treated syringe. This blood was used for *in vitro* "heparin tolerance" tests, prothrombin and thrombin clotting times, and platelet, hematocrit, and fibrinogen determinations. Five and 28 minutes later other blood specimens were obtained and these tests repeated. Thus, three separate control determinations were made before the beginning of the thromboplastin infusion. One minute after the last control specimen had been drawn, an infusion of dog brain thromboplastin was started in the left saphenous vein through a 20 gauge needle. This particular preparation of dog brain thromboplastin contained 0.64 mg. of brain solids per ml. The thromboplastic activity of the material was such that it gave a prothrombin time of 18 seconds with the dog's plasma. The infusion was maintained at a rate of 0.75 ml. per minute for a period of eight minutes at which time another specimen of blood was obtained for study.

The infusion was continued at the following rates during each successive period: 1.0 ml. per minute for nine

[3] Heparin sodium was kindly supplied by the Upjohn Company, Kalamazoo, Michigan.

[4] Thrombin (bovine) was obtained through the courtesy of the Upjohn Company, Kalamazoo, Michigan.

[5] The thromboplastin gave a prothrombin time of 22 seconds with the dog's plasma.

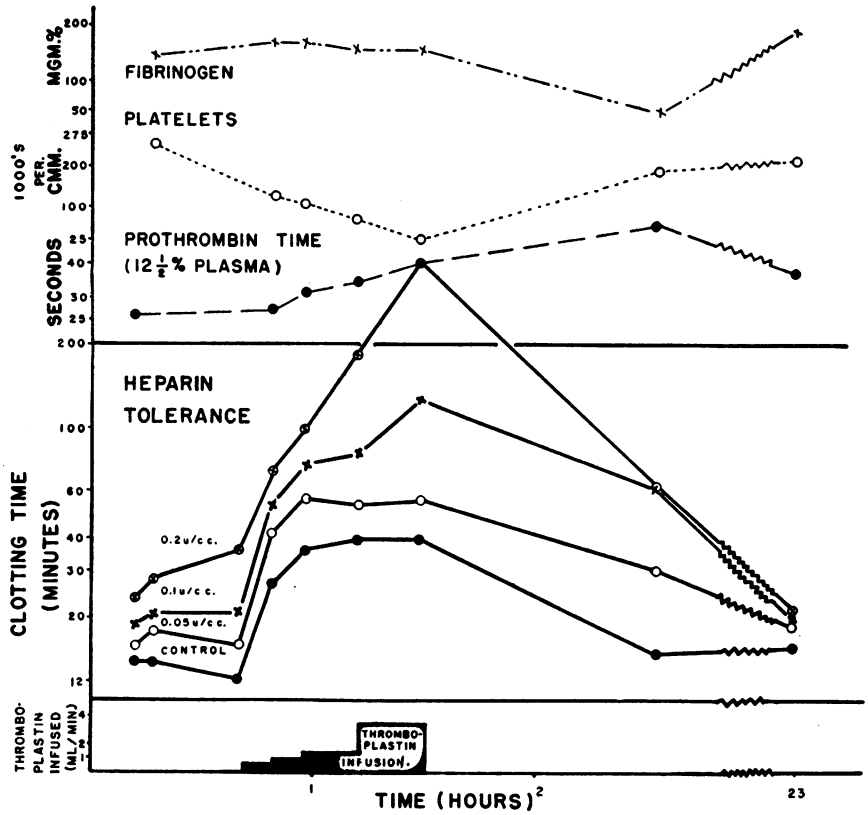

FIG. 1. EFFECT OF THROMBOPLASTIN INFUSION ON HEPARIN TOLERANCE

minutes, 1.5 ml. per minute for 14 minutes, and 3.2 ml. per minute for 18 minutes. At the end of each period blood specimens were obtained for clotting studies. The total time required for the administration of thromboplastin was 49 minutes. Blood drawn during the infusion did not clot after the addition of oxalate, indicating that thrombin was not present in the blood obtained from the jugular vein. One and 23 hours after the infusion was discontinued, additional specimens were obtained.

The animal withstood the procedure well. Pulse and respirations remained normal, and at no time was there evidence of distress. Recovery from anethesia was uncomplicated, and the dog appeared normal for the period of observation of one week following the procedure.

In this experiment the infusion of thromboplastin resulted in prolongation of the clotting time, exaggerated response to heparin, prolongation of the prothrombin times of undiluted and 12½% plasma, and decrease in platelets. These changes could not be accounted for on the basis of alteration in fibrinogen concentration. The hematocrit values did not change appreciably.

It was apparent that the infusion of the material used in this experiment did not increase heparin resistance but actually led to the development of a severe coagulation defect. Therefore, in other experiments thromboplastin preparations of lower activity were employed. In one experiment the thromboplastic activity of the material infused was such that it gave a prothrombin time of 30 seconds with the dog's plasma. Eighty and one-half ml. of this preparation were given at increasing rates over a period of 66 minutes. There was slight prolongation of the clotting time, exaggerated response to heparin, increase in the prothrombin time of 12½% plasma, and decrease in platelets. At no time was a reduction of the fibrinogen concentration detected.

When the amount of thromboplastin infused was further decreased, the effects on the coagulation mechanism were much less striking. An experiment was performed in which 31 ml. of a brain preparation were infused over a period of 33 minutes. This thromboplastin gave a prothrombin time of 27 seconds with the dog's plasma. The results were similar to those obtained in the

previous experiments except that the changes were slight and of questionable significance.

In another experiment the effects of a prolonged infusion of very weak thromboplastin preparation were studied. This thromboplastin gave a prothrombin time of 61 seconds with the dog's plasma.[6] A total of 89 ml. was given over a period of 124 minutes at an average rate of infusion of 0.72 ml. per minute. There was no evidence of hypercoagulability of the blood or of increased resistance to heparin. The slight changes which occurred suggested a mild impairment of coagulation.

Since it was not possible to demonstrate hypercoagulability of the blood during the infusion of relatively weak thromboplastin, attempts were made to produce temporary states of hypercoagulability by the injection of very potent material. One dog received an infusion of 28 ml. of a thromboplastin which gave a prothrombin time of 12 seconds with the dog's plasma. This material contained 6.9 mg. of brain solids per ml. The infusion was given over a period of 52 minutes. All of the changes depicted in Figure 1 were also seen in this experiment, but were more marked. However, the animal withstood the infusion without evidence of disability.

In other experiments potent thromboplastin was injected at a rapid rate from a syringe. Table I summarizes the results of an experiment of this type. Ten ml. of a preparation which gave a prothrombin time of 22 seconds with the dog's plasma were injected within a period of 30 seconds. This material contained 2.3 mg. of brain solids per ml. Blood drawn one minute after the injection showed slight prolongation of the clotting time and a greatly exaggerated response to heparin. The clotting time quickly returned to normal, but marked sensitivity to heparin persisted. No significant change occurred in the prothrombin times. There was no reduction of the fibrinogen concentration. The platelet count fell precipitously but returned to normal within 33 minutes. There was no external evidence of intravascular clotting, and the animal appeared to suffer no adverse effects from this injection. Thirty-four minutes after the first injection, 10 ml. of a preparation which gave a prothrombin time of 13 seconds were injected within 30 seconds. This amount of brain extract caused immediate death in animals which had not received a prior injection of less concentrated material. Although the respiratory rate increased from 12 to 35 per minute and respirations appeared labored for about 15 minutes after the injection, the animal survived the procedure without further difficulty. Recovery from anesthesia was uneventful, and the dog was normal in behavior and appearance for the period of observation of one week.

[6] The feeble activity of this thromboplastin was indicated by the fact that this dog's plasma clotted in 90 seconds when 0.85% saline solution was substituted for the thromboplastin preparation.

TABLE I

Effects of rapid intravenous injection of thromboplastin

Time in relation to thromboplastin injection	Clotting times of 1.0 ml. portions of blood containing the following concentrations of heparin (units per ml. of blood)				Prothrombin time		Fibrinogen	Platelets
	0	0.10	0.15	0.20	Undiluted plasma	12½% plasma		
	min.	*min.*	*min.*	*min.*	*sec.*	*sec.*	*mg. %*	*per cmm.*
61 minutes before	16	24	27	—	—	—	234	300,000
58 minutes before	16	24	34	—	—	—	264	283,000
26 minutes before	11	23	26	75	8	30	—	—
Thromboplastin* 10 ml. injected within 30 seconds								
1 minute after	20	220	270	>420	9	32	274	96,000
3 minutes after	17	50	240	>420	9	33	366	102,000
5 minutes after	14	25	110	>420	8	33	254	107,000
33 minutes after	13	74	190	210	8	33	397	287,000

* This material gave a prothrombin time of 22 seconds with the dog's plasma.

The rapid injection of 10 ml. of potent thromboplastin killed the animals unless they had been rendered refractory by previous injection of diluted thromboplastin. When dogs were given rapid intravenous injections of 10 ml. of very potent thromboplastin, there was immediate profound respiratory distress and death followed within several minutes. Blood drawn within 30 to 60 seconds following the injection clotted before ejection from the syringe. Clots formed even when the blood was oxalated, indicating the presence of thrombin. In some experiments the rapid injection of sublethal amounts of thromboplastin was followed by evidence of thrombin activity in the blood drawn within a few seconds after the injection. However, blood drawn one minute or more after the termination of the thromboplastin injection no longer showed the presence of thrombin.

In vivo "heparin tolerance." The effect of infusion of thromboplastin on the response to intravenous heparin is shown in Figure 2.

A healthy 7.3 kilogram mongrel female dog, anesthetized with nembutal, was given an injection of 0.9 ml. of a heparin solution containing 100 units per ml. into the saphenous vein. Blood for clotting studies was drawn before the injection and two, five, 10, 20, and 30 minutes thereafter. After the clotting time had returned to normal, an intravenous infusion of 31 ml. of thromboplastin was started in the contralateral saphenous vein. The thromboplastic activity of this material was such that it gave a prothrombin time of 30 seconds with the dog's plasma. The infusion was continued for a period of 40 minutes at an average rate of 0.75 ml. per minute. Five minutes after the infusion was started, another specimen of blood was obtained for clotting studies. Then a second injection of the same amount of heparin which had been previously employed was given. Specimens for clotting studies were obtained at the same intervals as after the control injection of heparin.

There was no increase in resistance to the anticoagulant activity of the injected heparin during

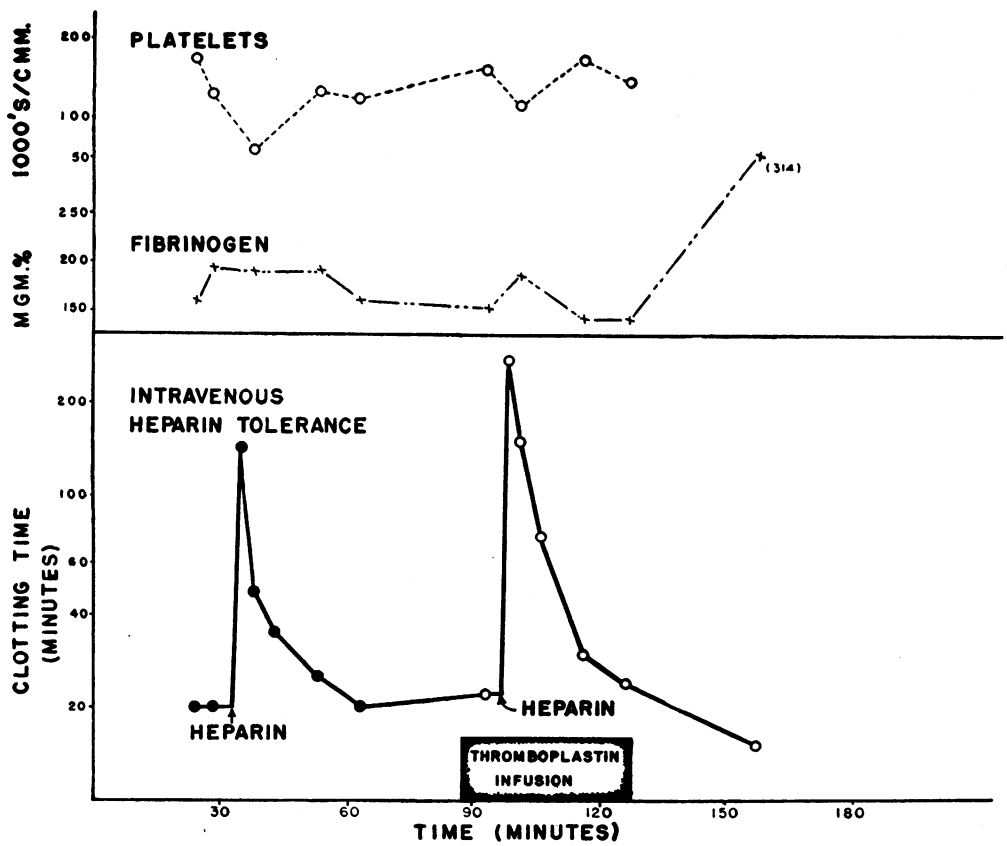

FIG. 2. EFFECT OF THROMBOPLASTIN INFUSION ON *IN VIVO* HEPARIN TOLERANCE

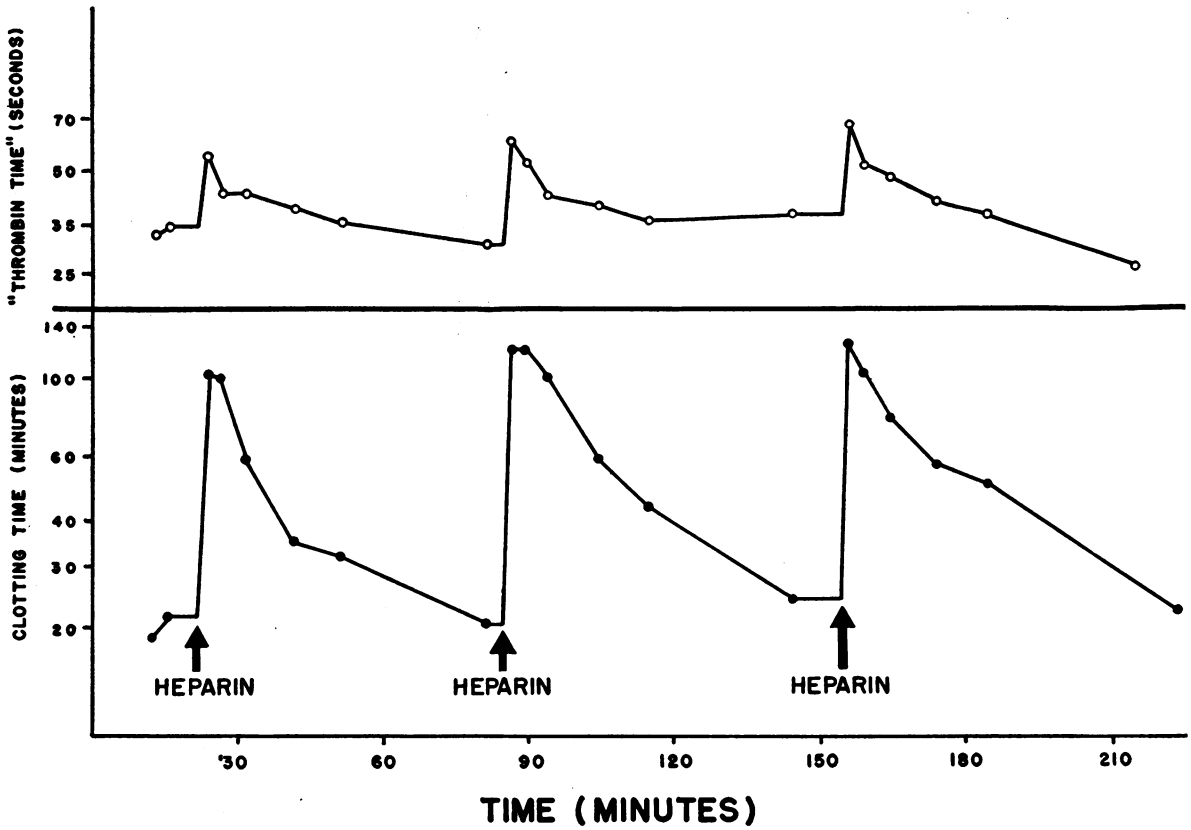

FIG. 3. EFFECT OF REPEATED INJECTIONS OF HEPARIN ON CLOTTING AND "THROMBIN" TIMES

the infusion of thromboplastin. In fact, the clotting times were longer than after the control injection of heparin. No significant change in fibrinogen levels occurred until the end of the experiment, at which time there was an increase. The platelet count fell after the control injection of heparin, but the fall was not appreciable when heparin was injected during the thromboplastin infusion. In a control experiment, repeated intravenous "heparin tolerance" tests were performed

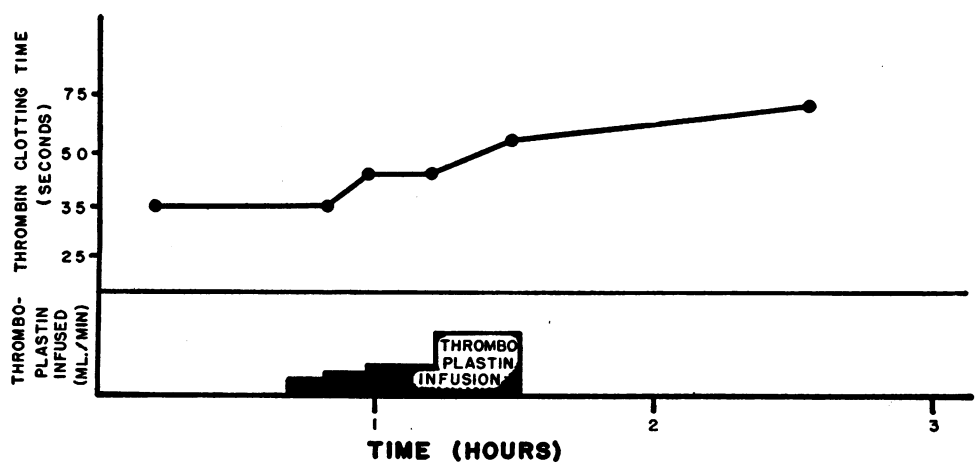

FIG. 4. EFFECT OF THROMBOPLASTIN INFUSION ON "THROMBIN TIME"

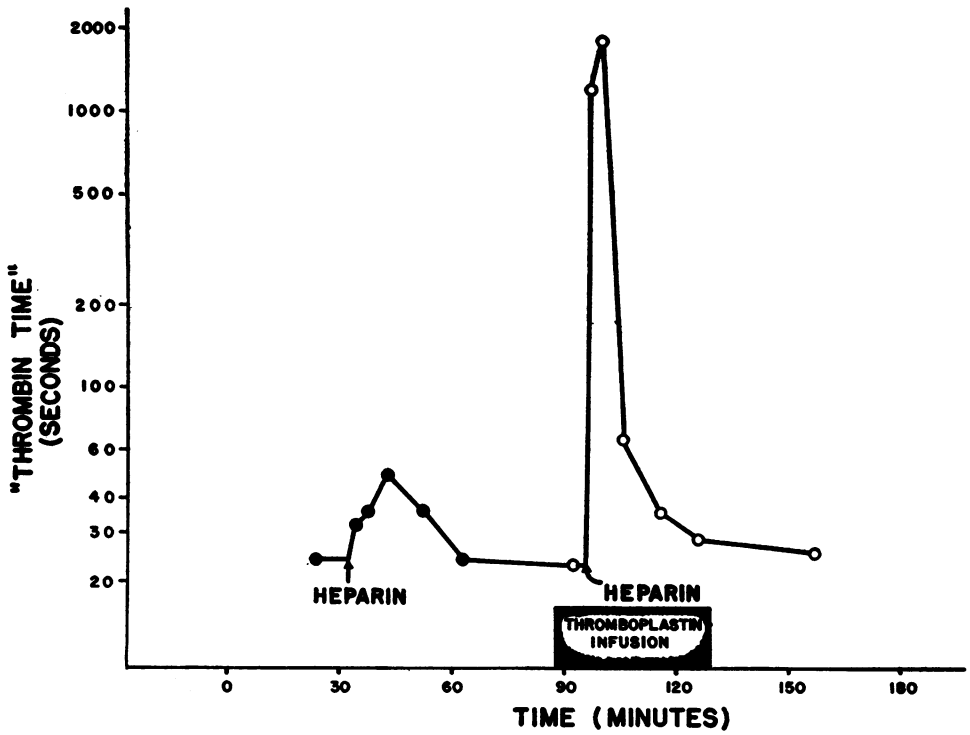

FIG. 5. EFFECT OF THROMBOPLASTIN INFUSION ON "HEPARIN TOLERANCE" AS MEASURED BY "THROMBIN TIMES"

on an anesthetized dog to which no thromboplastin was administered. Results of this experiment, shown in Figure 3, demonstrate the reproducibility of the "heparin tolerance" test under these conditions.

"Heparin tolerance" as measured by the thrombin clotting time. The thrombin clotting time of oxalated plasma, obtained after the intravenous injection of heparin, has been used to measure "heparin tolerance" (9). In the experiment described in Figure 1 the thrombin clotting time was prolonged during and after the infusion of thromboplastin (Figure 4). When smaller amounts of thromboplastin were administered, there was no change in thrombin clotting times during or after the infusions.

In the experiment depicted in Figure 2 thrombin clotting times were determined after the two intravenous injections of heparin. The results are shown in Figure 5. The injection of heparin alone prolonged the thrombin clotting time. The same amount of heparin injected during the thromboplastin infusion resulted in extreme prolongation. That this prolongation was not due to the additive effects of repeated injections of heparin is shown in a control experiment (Figure 3). Thus the infusion of thromboplastin produced no increase in resistance to heparin as measured by the thrombin clotting time.

DISCUSSION

"Heparin tolerance" tests have been employed clinically to detect hypercoagulability of the blood and tendency to intravascular clotting. However, there is no experimental evidence that these tests actually measure hypercoagulability of the blood, nor have the factors increasing heparin resistance been studied. A systematic study of factors which influence the anticoagulant effect of heparin has been carried out in this laboratory. When thrombin formation is impaired, blood is rendered more sensitive to the anticoagulant action of heparin. The concentration of heparin required to delay the coagulation of blood is directly related to the platelet concentration (10). No evidence was found that platelet-free plasma was capable of inactivating heparin *in vitro*. Platelet-free plasma from normal subjects as well as from patients

with thrombotic or hemorrhagic disease did not affect the anticoagulant activity of heparin (11).

It has been suggested that liberation of thromboplastin into the blood may render the blood hypercoagulable and predispose to thrombosis (3, 4, 9). The "heparin tolerance" test has been thought to be a measure of circulating thromboplastin (3–5, 9). The experiments described provide no support for this belief. Dogs receiving thromboplastin infusions, with the rate of infusion and thromboplastic activity of the material varied over wide ranges, displayed no evidence of hypercoagulability of the blood. On the contrary a coagulation defect associated with increased sensitivity to heparin was observed. Rapid injection of potent thromboplastin resulted in the formation of gross clots in the right side of the heart and pulmonary arteries, and death of the animal in a few minutes. Blood drawn from these animals within a few seconds following the injection clotted even though oxalated, suggesting that thrombin was present in the circulating blood. In some similar experiments in which the animal survived, oxalated blood clotted. This phenomenon was observed only in blood drawn within a few seconds after the rapid injection of potent thromboplastin.

When large amounts of thromboplastin were infused, there was a reduction of the plasma fibrinogen concentration. Complete removal of fibrinogen can be accomplished *in vivo* by thromboplastin infusion (12, 13). When smaller amounts were administered so that the fibrinogen level was not appreciably altered, a severe coagulation defect was nevertheless produced. This defect was characterized by increased sensitivity to the anticoagulant action of heparin, prolongation of the clotting time, decrease in the platelet count, prolongation of the prothrombin time and in particular the prothrombin time of diluted plasma, and delayed coagulation of plasma by thrombin. The degree of the coagulation defect was related to the amount of thromboplastin administered and the rate of the infusion.

The nature of this peculiar coagulation defect is not completely understood. Thrombin formation was undoubtedly impaired by the reduction in the concentration of platelets and possibly in prothrombin. However, the prolonged thrombin clotting time cannot be explained on this basis. It has previously been shown that the plasma of dogs infused with thromboplastin may acquire thrombin-inhibitory properties (13). The degree of thrombocytopenia produced was not adequate to account for the increased sensitivity of the blood to heparin.

Some investigators have noted that animals may be protected from an otherwise lethal injection of thromboplastin by prior injection of a sublethal dose. This refractory phase has been attributed to the removal of fibrinogen by the first injection (12, 14). The protective action of a preliminary sublethal injection was confirmed. However, it is noteworthy that this refractory state occurred even though the fibrinogen concentration was not reduced by the first injection.

It has been thought by some observers that shortening of the prothrombin time of diluted plasma is an index of tendency to thrombosis (15, 16). In the experiments recorded here the prothrombin times of diluted plasma were not shortened by the intravenous infusion of thromboplastin. On the other hand there was frequently a prolongation of the prothrombin time of diluted plasma.

The intravenous injection of heparin resulted in a decrease in platelet concentration (Figure 2). The thrombocytopenia produced by intravenous injections of commercial heparin has been reported by others (17, 18). However, these investigators used larger amounts of heparin than were used in the present experiments. It is of interest that small amounts of heparin also produced transient thrombocytopenia.

SUMMARY

1. The effect of the intravenous infusion of homologous brain thromboplastin on the "heparin tolerance" test was studied in dogs. No increase in resistance to the anticoagulant activity of heparin was noted during the infusion of thromboplastin. On the other hand exaggerated response to heparin was observed.

2. The infusion of thromboplastin resulted in a coagulation defect which was characterized by prolongation of the clotting time, prolongation of the prothrombin time of diluted and undiluted plasma, decrease in platelet concentration, and prolongation of the thrombin clotting time. These effects

occurred before there was a measurable reduction in the fibrinogen concentration.

3. No evidence was found to support the view that the "heparin tolerance" test is a measure of circulating thromboplastin.

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