

Effect of Extracorporeal Enzymatic Deheparinization on Formed Blood Components

*Annette K. Larsen, *Robert J. Linhardt, †‡David Tapper, §Michael Klein, and *†||Robert Langer

**Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge; †Department of Surgery, Children's Hospital Medical Center, Boston; ‡Harvard Medical School, Boston, Massachusetts; §Department of Surgery, Children's Hospital of Michigan, and Wayne State University School of Medicine, Detroit, Michigan; and ||Harvard-Massachusetts Institute of Technology Division of Health Sciences and Technology, Boston, Massachusetts, U.S.A.*

Abstract: A stirred blood filter containing an immobilized enzyme, heparinase, has been used to neutralize heparin's anticoagulant activity at the outflow of an extracorporeal circuit in dogs. The hematocrit and red blood cell count remained unchanged throughout the 90-min perfusion period. Platelet and white blood cell counts decreased early in the procedure to ~20% of the initial levels, but then returned to 30 and 70%, respectively, of their initial values by the end of the procedure. After 24 h normal levels were reestablished. In vitro experiments with human blood were conducted to determine the prin-

cipal cause of the observed decrease of formed blood components. An unstirred heparinase filter preserved platelets and white blood cells better than stirred filters possessing higher, the same, or no heparin-degrading capacity, suggesting that most of the loss of formed blood components is due to stirring and not to the heparinase or the Sepharose support on which the enzyme is immobilized. **Key Words:** Enzymes—Extracorporeal circulation—Blood—Deheparinization—Heparin—Heparinase—Immobilized enzyme.

When blood encounters the surface of extracorporeal medical devices such as the artificial kidney or the pump oxygenator, complex interactions leading to thrombosis occur (1). Therefore, an anticoagulant, heparin, is systemically administered concomitant with the operation of such devices. However, heparin usage often leads to complications such as bleeding and platelet dysfunction (2).

A blood filter containing immobilized heparinase, a heparin-specific enzyme (3) that degrades heparin to small saccharides (4,5), has been developed. This filter could be placed at the outflow of any extracorporeal device, thereby permitting full heparinization of the blood entering the extracorporeal device without simultaneously heparinizing the pa-

tient systemically. Although this filter has proven efficient in rapidly clearing heparin from blood (6), it is critical to study the effects of the filter on formed blood components, since the effects on white blood cells and platelets are a general concern in extracorporeal circulation. In this article the effect of the filter on hematological parameters has been examined, with particular attention to heparinase activity, and mixing conditions within the filter.

MATERIALS AND METHODS

Heparin (grade II, 153 USP U/mg) as the sodium salt from porcine intestinal mucosa was purchased from Sigma Chemical Co. Vacutainers containing either citrate or EDTA were from Becton Dickinson. Thrombafax reagent was from Ortho Diagnostic Systems, Inc. (Raritan, NJ, U.S.A.). Polypropylene disposable columns (5 ml) were from Biorad, Inc. (Richmond, CA, U.S.A.). Sodium pentobarbital (Nembutal) 50 mg/ml was obtained from Abbott Laboratories (Chicago, IL, U.S.A.). Seph-

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Dr. Linhardt's present address is College of Pharmacy, University of Iowa, Iowa City, IA 52242, U.S.A.

Dr. Tapper's present address is Children's Orthopedic Hospital, University of Washington, Seattle, WA 98105, U.S.A.

Address correspondence and reprint requests to Dr. Langer at Massachusetts Institute of Technology Division of Health Sciences and Technology, Cambridge, MA 02139, U.S.A.

arose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Blood samples were centrifuged on a Beckman refrigerated centrifuge (model no. TJ-6). Complete blood count was performed on a Coulter Counter by the hematology laboratory at Brigham and Women's Hospital (Boston, MA, U.S.A.). Normal human blood was obtained from the Children's Hospital Blood Bank (Boston, MA, U.S.A.). Donors were healthy and advised not to eat the morning prior to giving blood. All donors were blood type A⁺. The blood was drawn into 63 ml of phosphate-dextrose solution containing 21 mg heparin corresponding to 6 U/ml heparin. The blood was stored at room temperature until the in vitro experiments could be performed, which were performed at the most within 2 h after blood collection.

Enzyme preparation

Heparinase was prepared from *Flavobacterium heparin* (7,8). After cell sonication it was purified with protamine sulfate and hydroxylapatite chromatography (9). At this stage of purification, heparinase has a specific activity of 150 mg heparin degraded/mg protein/h.

Enzyme immobilization

Heparinase was immobilized on Sepharose 4B beads at a concentration of 1 mg protein/ml beads using a variation (5) of the procedure of March et al. (10). The activated support was washed with at least 10 volumes of distilled water to ensure that none of the immobilization reagents remained. After the immobilization procedure, the support was further washed with 0.5 M NaCl, 0.25 M phosphate buffer, pH 7. Low-activity immobilized heparinase was prepared by heating immobilized heparinase at 100°C for 5 min. This reduced the activity to 20% of its initial value. Completely inactive immobilized heparinase was prepared by heating the immobilized heparinase at 100°C for 30 min.

Activity of immobilized heparinase

In buffer solution

The activity of the immobilized enzyme was assayed by incubating the Sepharose 4B-heparinase beads in a 250 mM sodium acetate solution, pH 7.0, containing 8.3 mg/ml heparin at 30°C. The disappearance of heparin was determined spectrophotometrically by an Azure A dye-binding assay (6), and the appearance of the degradation products was determined by measuring the absorbance at 232 nm (4).

In blood

Blood samples were drawn into citrated Vacutainers and immediately placed on ice. Plasma was prepared by centrifugation at 3,000 rpm for 20 min. The anticoagulant activity of heparin in plasma was measured by the activated partial thromboplastin time using Thrombofax reagent (11,12). All experimental values were compared with a standard curve generated from citrated plasma heparinized with from 0 to 0.5 USP U/ml.

Measurement of formed blood components

Samples for red blood cell counts, white blood cell counts, platelet counts, hematocrit, and hemoglobin were collected in EDTA-containing Vacutainers and analyzed immediately with a Coulter Counter.

Heparinase filter

The heparinase filter was prepared as described (6). Two holes, 9.5 mm in diameter, were made in the top of a Bentley infusion filter (model PFT-100). Silicone rubber tubing (Cole-Parmer; inner diameter 0.8 cm; outer diameter 1.1 cm; length 27 cm) was inserted 10 cm into one hole and 3 cm into the other hole, and the connections were sealed with a silicone sealant. This is the system through which the Sepharose-heparinase beads are recirculated. A 40-cm silicone tube (Cole-Parmer; inner diameter 1.0 cm; outer diameter 1.5 cm) with a sample port was clamped to the input of the filter. A medical-grade Tygon tube (Cobe Hemaflow) from a kidney dialysis set with a bubble trap and a sampling septum was connected to the filter exit. The reactor was filled with saline and then purged of all air bubbles. A suspension of the Sepharose-heparinase was then slowly (10–15 ml/min) pumped into the reactor.

The blood flows through the filter at a flow rate of 50 ml/min, the flow rate normally employed during pediatric hemodialysis (13,14). When blood enters the filter, it is thoroughly mixed with the immobilized enzyme. The blood readily flows through the filter, whereas the immobilized heparinase remains because the beads on which the enzyme is immobilized are too big to penetrate the filter. The content of the blood filter is stirred by recirculating the beads through the filter at a flow rate of 300 ml/min using a peristaltic pump ("stirred flow system"). This prevents the beads from settling. An "unstirred flow system" was created by turning off the recirculating pump.

Changes of formed blood components in a nonflow system

Heparinized (6 U/mL), citrated (3.8% sodium citrate/whole blood, 1:10 vol/vol) human blood in Va-

cutainer tubes was incubated for 30 min at 37°C with either saline, Sepharose 4B, free heparinase (400 µg/ml), heat-denatured immobilized heparinase, or active immobilized heparinase. The ratio of bead volume to blood volume was 1:2. After a 30-min incubation period, the blood was filtered through a 5-ml polypropylene disposable column to remove the Sepharose beads. Samples not containing beads were also filtered. The blood was then drawn into EDTA-containing Vacutainers for complete blood counts. All samples were tested in triplicate.

In vitro deheparinization

The heparinase filter was used with a reservoir consisting of 2 units of freshly drawn heparinized (6 U/ml) human blood. The blood was passed from the reservoir through the filter and then returned to the same reservoir at a flow rate of 50 ml/min. The recirculating pump was used during all of the experiments except for one involving an unstirred filter in which the pump was turned off. Samples were taken from the reservoir throughout the experiments. These experiments were done in duplicate.

In vivo deheparinization

Five dogs weighing 11–22 kg were anesthetized by intravenous injection of Nembutal (10 mg/kg body weight). The common carotid artery and internal jugular vein were cannulated with a Scribner shunt (15) and connected to the filter with large-bore Silastic tubing (6). Heparin (450 U/kg body weight) was administered intravenously through a separate peripheral vein catheter. Blood was withdrawn from the carotid artery by a peristaltic pump attached to the filter. The effluent blood from the filter was passed through a bubble trap containing a Dacron mesh thrombus filter and returned to the animal through the jugular vein (6). Blood samples were taken from an indwelling catheter in the femoral artery.

RESULTS

Blood deheparinization

In the in vitro flow experiments using stirred blood filters containing active Sepharose-heparinase, 80% of the anticoagulant activity of heparin was degraded after 1 reservoir volume had passed through the filter (20 min), and 90% was degraded by the time the 3 reservoir volumes had circulated. Control filters packed with Sepharose beads or heat-inactivated immobilized heparinase showed no change in heparin activity (Fig. 1).

In the in vivo experiments, one pass (20–30 min) through the stirred heparinase filter of the dogs'

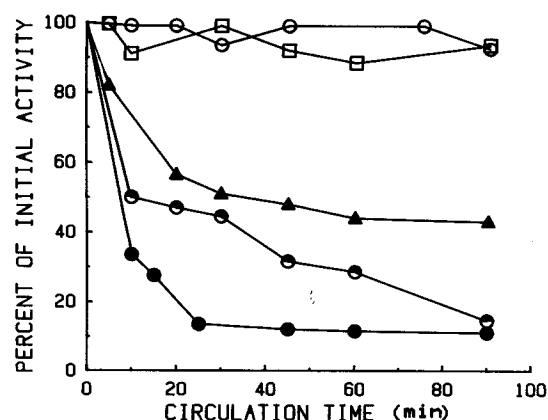


FIG. 1. In vitro deheparinization of human blood. Heparin activity within a reservoir containing 2 units of heparinized (6.3 U heparin/ml) human blood circulating (50 ml/min) through the heparinase filter. The percent of initial activity as measured by activated partial thromboplastin time is plotted versus time. The filter was loaded with either Sepharose-heparinase (filled circles), low-activity Sepharose-heparinase (half-filled circles), heat-inactivated Sepharose-heparinase (open circles), or Sepharose (squares) and stirred by a recirculating pump. The filter was also examined with Sepharose-heparinase without stirring (triangles). The total volume of the reservoir passed through the filter every 20 min.

total blood volume degraded 80% of the anticoagulant activity of heparin as determined in blood samples taken from the dogs. By the third pass 99% of the heparin was degraded (Fig. 2). These data provide further support for initial studies (6) showing that the heparinase filter can clear significant amounts of heparin in vivo and in vitro. Control experiments using blood filters with only Sepharose beads showed the same heparin levels as observed in control animals given heparin but not undergoing extracorporeal therapy (Fig. 2).

In both the in vivo and in vitro experiments, clot formation was seen near the outlet of the blood filter within 20–30 min in the blood filters with active heparinase. These filters, however, continued to support unrestricted flow even after 90 min when nearly all of the heparin, as measured by anticoagulant activity, had been degraded. When the blood filters were used without heparinase, only minimal clot formation occurred.

In vitro deheparinization and formed blood components

Heparinized, citrated human blood was incubated with heparinase, Sepharose beads, Sepharose-immobilized active heparinase, or Sepharose-immobilized heat-denatured heparinase at 37°C in a non-flow system and the formed blood components examined and compared with saline controls. The saline control and the heparinase sample had identical white blood cell ($6,400 \pm 200$, mean \pm SD)

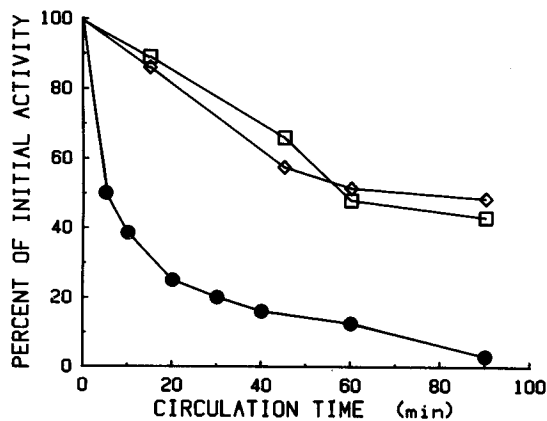


FIG. 2. In vivo deheparinization of canine blood. Dogs were given a bolus heparin dose of 450 U/kg, which results in a blood heparin concentration of 4 U/ml. Extracorporeal circulation at 50 ml/min was carried out through the heparinase filter. Anticoagulant activity as measured by activated partial thromboplastin time relative to the initial activity is plotted versus time. The filter was loaded with either Sepharose-heparinase (filled circles) or Sepharose (squares) and stirred by a recirculating pump at 300 ml/min. In addition, two control animals (diamonds) not connected to an extracorporeal circuit were heparinized at the same level and blood samples taken over a 90-min period. The total blood volume of the dogs passed through the filter approximately every 30 min.

and platelet ($199,500 \pm 5,500$) counts. The presence of Sepharose beads, however, led to a decrease of white blood cells ($5,800 \pm 100$) and platelets ($163,000 \pm 6,000$) whether the Sepharose was alone or had heparinase immobilized on it. The hematocrit (38%) and the red blood cell count ($4.60 \pm 0.04 \times 10^6$) were identical in all five groups.

The effect of in vitro deheparinization of human blood (Fig. 1) on formed blood components is shown in Fig. 3. White blood cell and platelet counts decreased with all the blood filter packings. The greatest loss occurred in the stirred filters with highly active Sepharose-heparinase, followed by the less active immobilized heparinase, the heat-inactivated heparinase, Sepharose alone, and finally the unstirred active Sepharose-heparinase filter. Most of the white cell and platelet loss occurred during the first 20 min in the stirred blood filters, whereas the loss occurred at an almost linear rate in the unstirred filter. No recovery of the white blood cells or platelets was observed during the 1.5 h of circulation through these filters under the in vitro conditions. Neither the red blood cell count nor the hematocrit showed any change.

In vivo deheparinization and formed blood components

The effect of in vivo deheparinization of canine blood (Fig. 2) on formed blood elements is shown

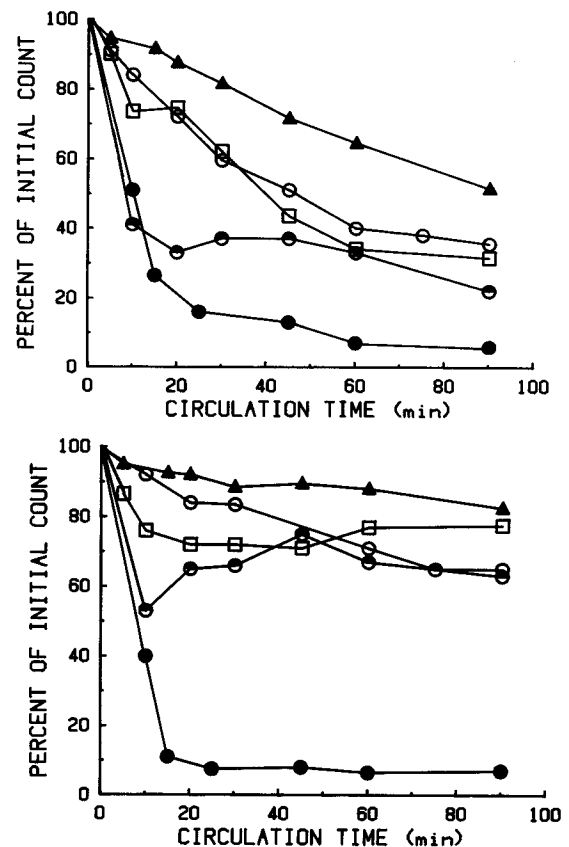


FIG. 3. Human white blood cell and platelet counts during in vitro circulation through the heparinase filter. The filter was loaded with either Sepharose-heparinase (filled circles), low-activity Sepharose-heparinase (half-filled circles), heat-inactivated Sepharose-heparinase (open circles), or Sepharose (squares). All these packings were stirred by a recirculating pump. The filter was also loaded with active Sepharose-heparinase and used without stirring (triangles). **Top:** White cells plotted as a percentage of the original count (100% = 5,700) versus time. **Bottom:** Platelets plotted as a percentage of the original count (100% = 220,000) versus time. Each point is the average of two experiments.

in Fig. 4. A slight decrease was observed in platelet and white blood cell levels when heparin was administered to the dogs that were not connected to an extracorporeal circuit. However, when the blood flowed through the stirred blood filter loaded with active immobilized heparinase, a greater decrease was observed in both white blood cell and platelet counts. There was a pronounced recovery in the white blood cell count and a moderate recovery in the platelet count after the initial decrease at the beginning of the extracorporeal circulation. Within 24 h of the conclusion of the experiment, the white blood cells and platelets had returned to normal levels. In all of the in vivo experiments, no decrease in the hematocrit and red blood cell count was observed.

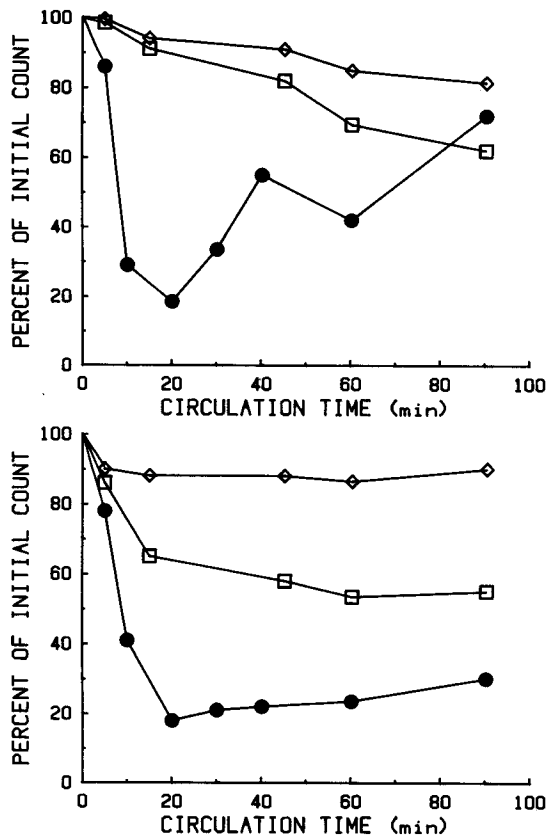


FIG. 4. Canine white blood cell and platelet counts during extracorporeal circulation through the heparinase filter. The blood filter was loaded and stirred with either Sepharose-heparinase (filled circles) or Sepharose (squares). In addition, results from a control experiment in which two dogs were heparinized but did not undergo extracorporeal circulation are shown (diamonds). **Top:** White cells plotted as a percentage of the original count (100% = 6,000) versus time. **Bottom:** Platelets plotted as a percentage of the original count (100% = 213,000) versus time. Each point is the average of two experiments.

DISCUSSION

The administration of heparin to dogs in the absence of any extracorporeal circulation causes a decrease in both white cell and platelet levels. This had been observed by others following heparin administration to both dogs (16-18) and humans (19,20). When human or canine blood is subjected to extracorporeal circulation, a further decrease in white cells and platelets occurs. This effect is greatest when a stirred blood filter with active immobilized heparinase is used. In the active stirred heparinase filter, an initial decrease of platelets and white blood cells to 20% of their initial levels occurred early in the *in vivo* procedure. The blood cells returned to 30 and 70%, respectively, of their initial values by the end of the 90-min procedure. Many reports describe a decrease in platelets and

white blood cells at the start of extracorporeal circulation, and the present results are similar to those generally obtained in extracorporeal circuits in dogs. For example, deLeval et al. (21) found a reduction of the platelets to 30-40% of their initial value within 5 min when dogs were placed on a bubble oxygenator. By 90 min the platelet levels were ~35% of their initial levels. The extracorporeal circuit without the oxygenator resulted in reduction of platelets to 55% of initial levels after 90 min of perfusion. Similarly, deJong et al. (22) found an initial decrease of platelets to 5% of their initial level within the first 5 min in an extracorporeal circuit with an artificial kidney, whereas the platelet count was 44% of the initial level in the same circuit without the kidney. After 90 min of perfusion, the platelet counts were 33 and 63% of their initial values, respectively. A decrease in the white blood cells to 20% of the initial level after 5 min of perfusion followed by an increase to almost normal levels after 1 h was observed by Toren et al. (23) in dogs connected to an artificial kidney.

A crucial question concerns the principal cause of the observed decrease in platelet and white cell counts. This could be (a) the mixing conditions in the filter; (b) the Sepharose beads; or (c) the heparinase.

Both the experiments with the unstirred filter (Fig. 3) and the *in vitro* deheparinization studies under nonflow conditions showed that the Sepharose beads contribute to only a minor part of the blood damage in the heparinase filter. Others have also demonstrated low levels of platelet retention on Sepharose supports (24).

Heparinase has apparently no effect on the formed blood components, as judged from the results of the nonflow experiments. This is consistent with the previous demonstration of the high specificity of this enzyme (3).

By implication, these results suggest that the mixing conditions are responsible. To investigate this further, experiments were conducted that demonstrated that an unstirred heparinase filter preserved platelets and white blood cells better than stirred filters possessing higher, the same, or no heparin-degrading capacity (Fig. 3). These results strongly suggest that the loss of formed blood components is due primarily to the stirring of the system.

The effect of stirring may be due to the release of mediators or the activation of serum factors in addition to purely physical damage. Figure 4 shows that after 10 min of extracorporeal circulation, *i.e.*, after approximately one-third of the blood has

passed through the highly active stirred heparinase filter, the white blood cell and platelet levels are not reduced by one-third, as would be expected if purely physical damage took place, but by 75 and 60%, respectively, suggesting that mediators may be involved. This is consistent with earlier reports (25) that indicated complement activation as one of the mechanisms contributing to the decrease of leucocytes in the peripheral bloodstream during extracorporeal circulation. Further indication of the lack of excessive physical damage in the filter is the fact that no hemolysis was observed.

The heparinase filter could be used either continuously during the entire operation to prevent high levels of heparin from ever entering the patient or at the very end of the perfusion to neutralize heparin similar to the way in which protamine is currently used. The specificity of heparinase and the fact that it is immobilized would eliminate the toxic effects that are sometimes caused by protamine (26).

Leaching of the immobilized heparinase from the support into the bloodstream may lead to immunogenic or toxic reactions. However, there was no leaching, as judged by protein concentration, from the support over a 1-month period at 25°C (6). Initial studies (6) showed no toxicity of the heparin degradation products. However, more extensive studies involving the clearance and toxicological effects of these products are currently being undertaken.

The results presented here suggest that a more blood-compatible filter may be developed by changing the flow characteristics of the system. The blood compatibility might also be further improved by decreasing the heparin-degrading capacity of the filter, thereby limiting clot formation.

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