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SCIENCE

An Enzymatic System for Removing Heparin in Extracorporeal Therapy

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Abstract. *The need to fully heparinize patients undergoing extracorporeal therapy often leads to hemorrhagic complications. To enable heparinization of only the extracorporeal circuit, a blood filter containing immobilized heparinase was developed. This filter degraded 99 percent of heparin's anticoagulant activity within minutes in both canine and human blood.*

Extracorporeal devices perfused with blood, such as the artificial kidney and the pump-oxygenator, have been an important part of the clinician's armamentarium for many years. These devices all rely on the patient being heparinized to prevent clotting in the device. However, heparinization of the patient often leads to hemorrhagic complications (*1*, *2*). With the prospect of longer perfusion times with machines such as the membrane oxygenator (*3*), the drawbacks of systemic heparinization are multiplied. A number of approaches have been tested to solve this problem. They include (i) administering protamine to neutralize heparin (*4*), (ii) using antithrombotic drugs other than heparin (*5*), (iii) bonding heparin, albumin, or fibrinolytic enzymes to the extracorporeal device (*6*), and (iv) developing new blood-compatible materials for construction of the device (*7*). Although these approaches have led to some improvements, control of the heparin concentration in the blood remains a serious problem.

We suggest a novel approach that would permit full heparinization of blood entering any extracorporeal device but which would enable enzymatic elimination of the heparin before the blood returned to the patient. This approach consists of placing a blood filter containing immobilized heparinase—which degrades heparin into small polysaccharides—at the effluent of the extracorporeal device. Initial studies reported here to test the feasibility of this approach show that a conventional blood transfusion filter containing immobilized heparinase can remove the anticoagulant activity of clinically used amounts of heparin in several minutes in human blood and in dogs.

Heparinase was produced from *Flavobacterium heparinum* (*8*) and purified by cell sonication, nucleic acid precipitation with protamine sulfate, and hydroxylapatite chromatography (*9*). At this stage of purification, heparinase degrades 150 mg of heparin per milligram of protein per hour and is catalytically pure (free from contaminating sulfatases and glucuronidases) (*10*). Heparinase was covalently bound to Sepharose and washed to remove noncovalently bound enzyme by a variation (*9*) of the procedure of March *et al.* (*11*). There was no detectable leaching, as judged by protein concentration (*12*), of heparinase from the support over a 1-month period at 25°C.

Immobilization increases the stability of heparinase: at 4°, 25°, and 37°C the immobilized enzyme has half-lives of 5000, 1000, and 15 hours, respectively, compared with the native enzyme, which has half-lives of 125 and 30 hours and 1 hour at the same temperatures.

Active, immobilized heparinase and a control consisting of thermally inactivated, immobilized heparinase were loaded into two columns with a 1.5-ml bed volume. Heparin solutions were passed through both columns in stepwise fashion at concentrations of 15 to 75 μg/ml (clinically used heparin concentrations, having a specific activity of 0.15 U/μg, range from 5 to 25 μg/ml) (Fig. 1). As the heparin level in the input solutions increased, the difference in the heparin recovered at the outlets of the columns increased (Fig. 1). Even at 75 μg/ml, the heparin was largely removed by the column containing active heparinase, while the control column had no effect.

At flow rates above 1 ml/min the Sepharose packed tightly, preventing flow. Therefore we used a fluidized bed of Sepharose, obtained by pumping Sepha-

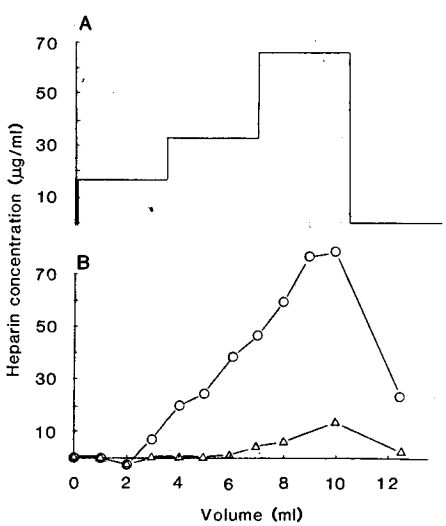

Fig. 1. (A) Input concentrations of heparin to both active and inactive columns, showing stepwise increases from 15 to 75 μg/ml. (B) Output concentrations of heparin from the column containing active heparinase (△) and the column containing heat-inactivated heparinase (○). Solutions of bovine serum albumin (60 mg/ml), salts, and heparin were passed through the columns (diameter, 1 cm) at a flow rate of 0.5 ml/min. The inactive heparinase was prepared by heating a suspension of active Sepharose-heparinase at 100°C for 30 minutes. The physiological concentrations of the nonheparin species in blood were used. Heparin concentration was measured by the azure assay (*8*, *17*).

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rose through a U-tube interconnected to a 100-ml blood transfusion filter at a flow rate six times greater than the rate at which blood was being pumped through the same filter (Fig. 2).

Whole human blood, anticoagulated with 90 U of heparin per milliliter (an even higher concentration than is normally used clinically) (*1*), was passed through the filter at a flow rate of 50 ml/min. Blood samples were taken directly from the blood reservoir (sample port a in Fig. 2) before and after each of six passes of the entire blood volume through the filter. As shown in Fig. 3A, 60 percent of heparin's anticoagulant activity, as measured by activated partial thromboplastin time (APTT) (*13*), was destroyed in 2 minutes (one pass), and almost all the activity was gone after 6 minutes (three passes). To demonstrate that the change in APTT was due to a decrease in the heparin concentration and not to a nonspecific effect of immobilized heparinase on coagulation factors, we also used an azure A assay that detects heparin metachromatically (*14*). This assay also showed that heparin levels fell as blood passed through the filter (Fig. 3A). Control filters containing Sepharose without heparinase showed no binding or degradation of heparin in the blood, as judged by both assays.

In an in vivo experiment, two dogs were anesthetized and the carotid artery and internal jugular vein were cannulated with a Scribner shunt (*15*) connected to Silastic tubing joined directly to the heparinase reactor. Heparin (450 U/kg) was added and blood was sampled after it left the filter (sample port b). Three heparin assays were used: APTT, whole blood recalcification time (WBRT) (*16*), and azure A (*8, 17*). Within 2 minutes

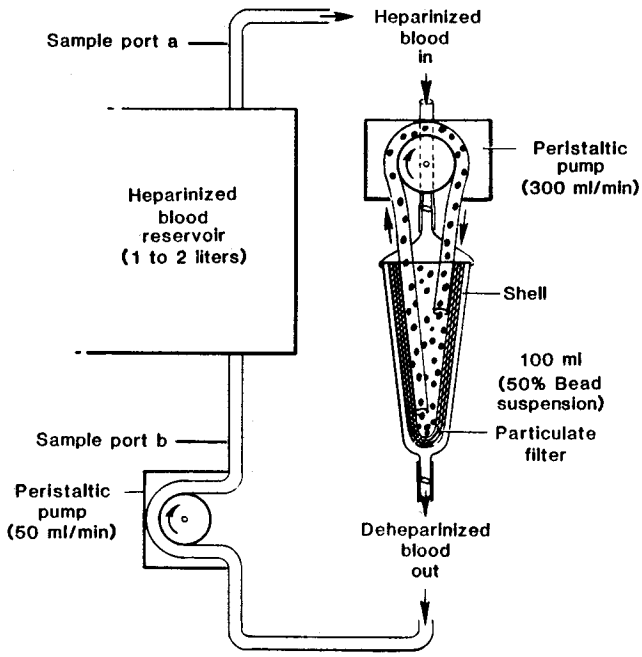

Fig. 2. Diagram showing the immobilized heparinase reactor system. Two holes 9.5 mm in diameter were made in the top of a Bentley infusion filter (model PFT-100). Silicone rubber tubing (inner diameter, 0.8 cm; outer diameter, 1.1 cm; length, 27 cm) was inserted 10 cm into one hole and 3 cm into the other hole and the connections were sealed with a silicone sealant. A 40-cm silicone tube (inner diameter, 1.5 cm; outer diameter, 1.0 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 to 15 ml/min) pumped into the reactor.

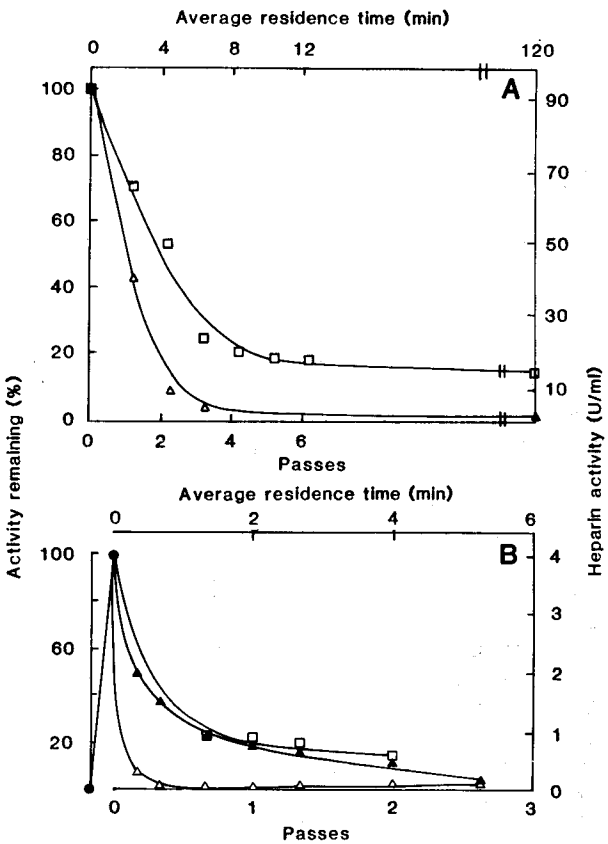

Fig. 3. (A) In vitro deheparinization of human blood. One liter of blood was obtained from healthy donors at the Children's Hospital Blood Bank, Boston. (The donors were advised not to eat during the morning of the donation or to take medication during the 3 days before donation.) While still fresh, the blood was heparinized (90 U/ml) and passed through the filter at a rate of 50 ml/min. The filter was placed between two 1-liter Fenwal blood transfer packs. After the entire blood volume from one pack had passed through the filter into the other pack (one pass), the packs were disconnected and their positions with respect to the filter were switched. The blood was sampled from the blood-containing pack before heparinization and after each of six passes through the filter. Samples were drawn into Vacutainers containing sodium citrate and centrifuged at 4500 rev/min for 20 minutes; the plasma was decanted from the formed components. Heparin in plasma was assayed for anticoagulant activity by APTT (△) (*13*) and for metachromatic activity by azure A (□) (*8, 17*). The reason that the azure A assay does not show zero heparin activity is that azure detects only heparin fragments of hexasaccharide size or larger. Fragments of this size are present [we determined this by high-pressure and gel permeation chromatography (*10*); digestion of heparin by heparinase in buffer also demonstrates this phenomenon], and these fragments are detected by azure A but do not possess anticoagulant activity as measured by APTT (*24*). (B) In vivo deheparinization of blood from an 11-kg female and a 22-kg male dog (blood volume, 80 cm^3/kg). A polyethylene catheter tip (No. 17 arterial, No. 19 venous) attached to a silicone rubber coupler tube was inserted into the carotid artery and internal jugular vein. The Tygon tubing from the filter (Fig. 2) was connected to these catheters. The dogs received heparin at a dose of 450 U/kg. Blood was drawn from the carotid artery through a peristaltic pump to the filter. Blood exiting the filter flowed through a sampling septum, then through a bubble trap containing a Dacron mesh thrombus filter, and was returned to the jugular vein. Samples were collected from sampling ports a and b in Vacutainers containing EDTA and analyzed immediately by Coulter counter at the Hematological Laboratory, Boston Brigham and Women's Hospital. The data were normalized to 0 percent activity before heparinization of the dog and 100 percent activity immediately after heparinization (●). Plasma samples to measure heparin in blood were prepared and assayed for anticoagulant activity by APTT [sample port a (▲) and sample port b (△)] and for metachromatic activity by azure A [sample port b (□)].

nearly all of heparin's anticoagulant activity measured by APTT was lost (Fig. 3B); the WBRT test gave identical results. The azure assay again demonstrated direct loss of heparin. Although it might be expected that removal of heparin would cause clotting, blood continued to flow unrestricted through the filter even after six passes. Blood samples were also taken at sample port a. After one pass there was an 80 percent loss of heparin by APTT (70 percent by azure) and after two passes there was a 90 percent loss by both assays (Fig. 3B). Heparin levels measured at sample port a were consistently higher than those measured at sample port b, presumably due to the mixing of blood in the dog. Control reactors without heparinase had no detectable effect on blood heparin levels in vivo (an in vivo half-life of 2 hours for heparin at this dosage was measured in the dog). The dogs still appeared healthy 3 months after the experiments. Blood was taken from the dogs 1 and 2 months after the experiments, and no antibodies to heparinase were detected by Ouchterlony precipitin tests (*18*). Products of the enzymatically degraded heparin were tested for cytotoxic and mutagenic effects on *Salmonella typhimurium* (*19*). No toxicity or mutagenicity was observed, even with concentrations 1000 times in excess of those we would anticipate clinically. In addition, heparin was neither toxic nor mutagenic by this assay.

At the end of the experiment, blood taken from the dogs showed no decrease in hematocrit, a 30 percent decrease in white blood cell count, and a 70 percent decrease in platelet count. These values are typical of those obtained for tests of extracorporeal circuits in dogs (*1*, *20*). To further improve the blood compatibility of immobilized heparinase, other heparinase supports and the use of a smaller filter should be examined. The latter is possible because heparinase has been purified to specific activities 70 times greater (with low recovery) than the enzyme used in this study by phosphocellulose ion-exchange chromatography and isoelectric focusing (*9*).

An immobilized heparinase filter could be used either at the end of a clinical procedure to eliminate heparin without the toxic effects of heparin-neutralizing substances such as protamine or continuously to prevent high levels of heparin from ever entering the patient. Blood filters as large as 2 liters are often used at the effluent of extracorporeal devices to remove microemboli. Heparinase might be bonded to the biomaterials of these filters. Unlike many proposed medical applications of immobilized enzymes such as asparaginase (*21*) and phenylalanine ammonia–lyase (*22*), immobilized heparinase would be used in procedures in which blood must enter extracorporeal circulation and in which existing biomaterials already interact with blood at the desired location (the end of the extracorporeal circuit). Thus this process may not require any additional apparatus or invasive procedure.

The heparinase filter may also be a prototype for other selective drug removal systems. Microbial selection techniques (*8*) could produce enzymes for use in filters to degrade potentially toxic drugs such as amphetamines or barbiturates. Such drug removal systems, like drug delivery systems (*23*), could prove useful in controlling drug concentrations in the blood.

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