

Heparinase: in vivo activity and immunogenicity in rabbits

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Anticoagulation with heparin is required during extracorporeal circulation for hemodialysis and cardiopulmonary bypass as well as during vascular surgery. Reversal of anticoagulation with protamine may be associated with hypotension and rebound anticoagulation and requires stoichiometric doses. Heparinase from *Flavobacterium heparinum* catalytically degrades heparin and reverses its anticoagulant effect. Heparin was administered to New Zealand White rabbits and plasma levels were assayed with the APTT anticoagulant assay and the azure A chemical assay. Heparinase actively degraded heparin both in vitro in rabbit plasma and in vivo in rabbit blood as determined by both the anticoagulant and chemical assays when compared to control heparin disappearance curves. Antibodies to heparinase were demonstrated by the ELISA technique in rabbits receiving i.v. heparinase. These antibodies, however, did not effect the activity of the enzyme in vitro or in vivo. No toxic effects of heparinase were noted in observations of the animals or in blood and histologic studies. Heparinase, either free or immobilized, may be a useful heparin-reversing agent without the drawbacks of protamine. (*J LAB CLIN MED* 102:828, 1983.)

Abbreviations: intramuscular(ly) (i.m.), intravenous(ly) (i.v.), activated partial thromboplastin time (APTT), enzyme-linked immunosorbent assay (ELISA), phosphate-buffered saline (PBS), sodium dodecyl sulfate (SDS), lactic acid dehydrogenase (LDH), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT)

Anticoagulation with heparin lasting several hours is required during extracorporeal circulation for hemodialysis and cardiopulmonary bypass as well as during vascular surgery. The particular operations and the underlying diseases for which they are performed lead to greater and more severe hemorrhagic complications than occur during heparinization at lower levels for treatment of pulmonary embolus and thrombophlebitis. Therefore reversal of anticoagulation with protamine, once extracorporeal circulation is stopped or the vascular anastomosis is completed, is generally performed. Protamine, however, is associated with hypotension¹ and has anticoagulant properties itself.² It may also be asso-

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ciated with a rebound phenomenon in which anticoagulant activity appears several hours after protamine administration.³ Heparinase (heparin lyase, E.C. 4.2.7) isolated from *Flavobacterium heparinum* acts very specifically on heparin and heparin-like linkages in heparin monosulfate.⁴ We have immobilized the enzyme in an active form to Sepharose 4-B (Pharmacia Fine Chemicals, Piscataway, N. J.) and have employed the immobilized enzyme in a fluidized bed reactor for the ex vivo removal of heparin from the blood.⁵ This device could permit regional heparinization of an extracorporeal device so that the patient would receive no heparin while the device remained fully heparinized.

In this study, we have examined the in vivo activity, immunogenicity, and toxicity of soluble heparinase to determine the consequences of leeching of enzyme from the reactor and to evaluate the free enzyme as a drug to reverse heparin at the conclusion of vascular surgery and extracorporeal circulation.

Methods

Heparinase. Heparinase was produced by fermentation of *Flavobacterium heparinum* in a defined medium in which heparin induces enzyme production, but glucose serves as the main carbon source for bacterial growth. Timely harvest just prior to the stationary phase of cell growth provides the maximum yield.^{6,7} Partial purification of heparinase was accomplished by protamine precipitation of nucleic acids and hydroxylapatite batch chromatography.⁴ This preparation demonstrates three major bands on SDS-gel electrophoresis. Molecular weight is approximately 50,000. There is no contaminating glucuronidase or sulfatase activity. The activity maximum occurs at pH 5.8 and the stability maximum occurs at pH 7.0. The maximum activity is at 30° C. The half-life of the enzyme at 30° is 25 hr.

Animals. New Zealand White rabbits, weighing approximately 2 kg, were anesthetized with xylazine hydrochloride (Rompun; Baynet Div. Cutter Laboratories, Shawnee Mission, Kans.), 0.45 mg/kg subcutaneously, and ketamine, 0.45 mg/kg i.m. Blood was obtained by catheterization of the right atrium via the right external jugular vein and then diluted 9:1 with 3.8 gm/100 ml sodium citrate. Plasma was obtained by centrifugation at 2000 rpm, 4° C, for 15 min. In the in vivo experiments, anesthesia was maintained and the catheter was left indwelling in the right atrium for drug administration and blood sampling.

Immunization. Heparinase was administered either i.v. in PBS or i.m. in 5 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) with one-half the dose given in each lateral thigh muscle.

Heparinase assay. Heparinase was assayed by assessing its effect on heparin. Beef lung heparin sodium injection U.S.P. (The Upjohn Co., Kalamazoo, Mich.) was added to pooled rabbit plasma at either 4 or 2 U/ml. Heparinase was added and vortex-mixed. The sample was incubated at 30° C for 1 hr. The APTT was immediately determined with the Platelin Activator Assay Kit (General Diagnostics, Morris Plains, N. J.). Results were recorded as units of heparin from a standard curve or as the natural log of the APTT in seconds (ln APTT) because this represents a more linear relationship to heparin concentration than the APTT.⁸

A colorimetric assay for chemical heparin was also performed after incubation of heparin (4 U/ml) in normal saline or rabbit plasma with heparinase for 1 hr at 30° C. One milliliter was added to 1 ml of azure A in distilled water (8 mg/100 ml) and absorbance at 620 nm was determined.⁹ The absorbance increases with decreasing heparin concentration. A standard curve was prepared each time the azure A assay was used and the results are reported in units of heparin.

Antibody assay. The ELISA was used to detect antibody formation.¹⁰ Heparinase was diluted to 5 µg/ml in a carbonate coating buffer and 200 µl was added to the wells in a polystyrene plate (Dynatech Laboratories, Inc., Alexandria, Va.). The plate was covered with Cellophane tape and incubated at 37° C for 1 to 2 hr. The wells were then emptied and washed with PBS with polysorbate 20 (Tween 20; ICI Americas, Inc., Wilmington, Del.). Rabbit serum to be tested (200 µl) was then added to each well in dilutions varying from 1:50 to 1:800 in PBS-Tween and incubated for 3 to 5 hr. The wells were emptied and washed with PBS-Tween. Goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Litton Bionetics, Inc., Kensington, Md.) was diluted 1:200 in PBS-Tween and 200 µl was added to each well. The plate was incubated at 37° C for 16 hr and then washed.

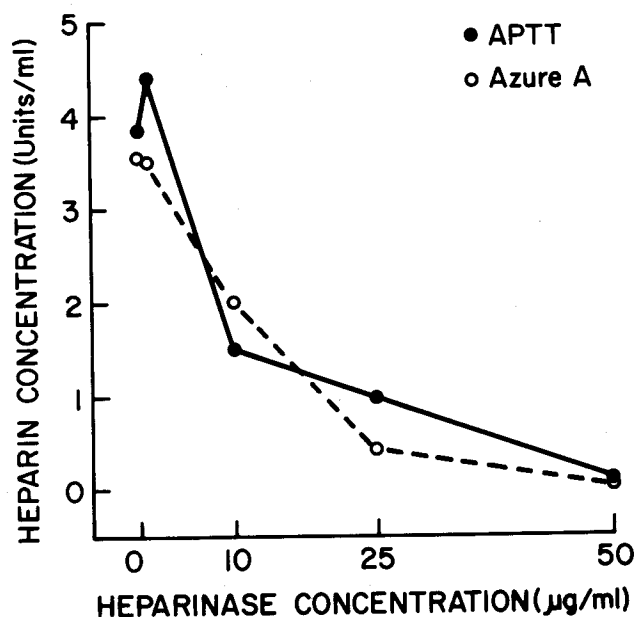


Fig. 1. In vitro dose-response curves for heparinase activity. Heparinase, 1, 10, 25, and 50 μg , was added to 1 ml of rabbit plasma to which 4 U of heparin had been added. The mixture was incubated at 30° C for 1 hr and heparin activity measured by both the APTT and azure A assays.

Alkaline phosphatase substrate in buffer containing 1 mg/ml *p*-nitrophenylphosphate disodium (Sigma Chemical Co., St. Louis, Mo.) was added to each well (200 μl) and allowed to react for 25 min at room temperature. The plate was then incubated at 37° C for 5 min and the reaction stopped by adding 4N NaOH. The absorbance in each well at 405 nm was then read in a Titertek Multiskan Type 310 C (Flow Laboratories, Inc., Rockville, Md.).

Pathology. Three rabbits who had received a total of at least 0.5 mg of heparinase over 1 week had blood samples drawn at 1 week and were sacrificed. Samples of liver, lung, heart, diaphragm, and small intestine were fixed in formalin, stained with hematoxylin and eosin, and examined by a veterinary pathologist. Blood samples were analyzed by the hospital pathology department for complete blood count, creatinine, blood urea nitrogen, sodium, potassium, chloride, carbon dioxide, bilirubin (total and direct), lipase, amylase, alkaline phosphatase, LDH, SGOT, and SGPT.

Results

Results of the in vitro assay of heparinase are depicted in Fig. 1. The activity of heparinase increased with its concentration until a maximum effect was reached. Different fermentations yielded enzyme with different activity per gram of protein. There was apparently denaturation of some enzyme in fermentations that were not perfectly controlled. There was equal purity on SDS-gel electrophoresis, and the curve of enzyme concentration vs. activity for enzyme from different fermentations had the same shape. Heparin removal curves from both the heparin activity assay (APTT) and the chemical heparin assay (azure A) were very similar.

The in vivo activity of heparinase is demonstrated in Fig. 2. A 50 $\mu\text{g}/\text{kg}$ concentration of heparinase given i.v. 2 min after 100 U/kg heparin resulted in more rapid disappearance of heparin than that in controls not receiving heparinase. The heparin concentration was significantly less ($p < 0.05$) at 5, 10, 15, and 20 min after heparin administration, 200

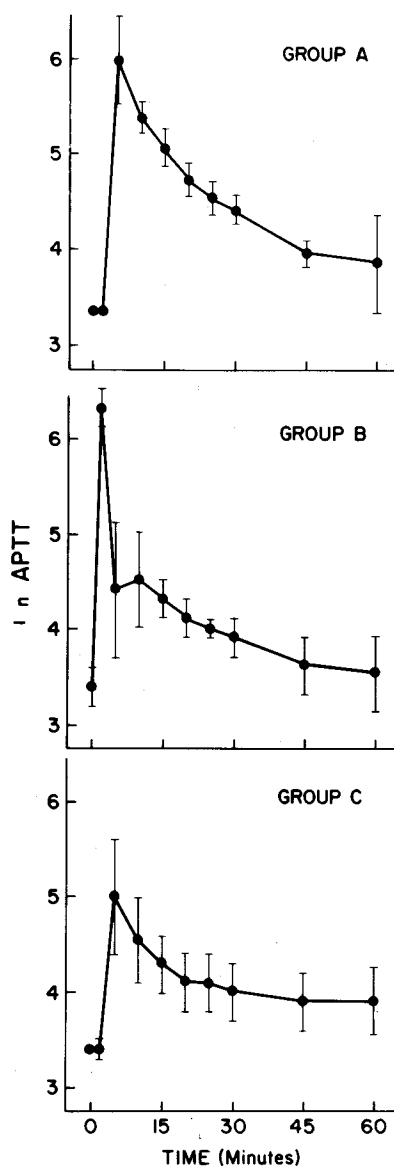


Fig. 2. In vivo heparinase activity in rabbits (mean \pm S.D., $n = 5$). Group A, Control animals given normal saline at 0 min and 100 U/kg heparin at 2 min. Group B, Heparin, 100 U/kg, given at 0 min and 50 μ g/kg heparinase at 2 min. Group C, Heparinase, 200 μ g/kg, given at 0 min and 100 U/kg heparin at 2 min.

μ g/kg heparinase had a similar effect. There was in vivo, a logarithmic concentration-to-activity relationship. In Fig. 3, the 1n APTT at 10 min after heparin administration as a percent of 1n control APTT is shown for three doses of heparinase given 2 min after heparin administration. The R was 0.737 with $p < 0.01$. All three are significantly different ($p < 0.5$) from the control where no heparinase was given. The 50 μ g dose gave a significantly ($p < 0.05$) different response from the 10 μ g dose.

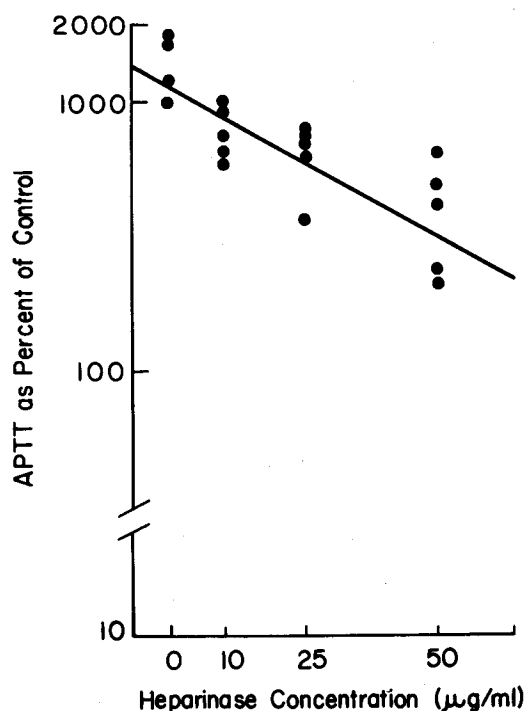


Fig. 3. In vivo dose-response relationship for heparinase in five rabbits. Heparinase was administered 2 min after 100 U/kg heparin. The 1n APTT is plotted as the percent of 1n control APTT. A semilog graph is used to demonstrate the logarithmic relationship.

Heparinase was immunogenic in rabbits (Fig. 4). Animals given 0.5 μg of heparinase i.m. in complete Freund's adjuvant on day 1 and in normal saline on day 21 demonstrated no antibody production by day 24. Those given 200 μg on a similar schedule, however, demonstrated minimal antibody production by day 8 and definite antibodies by day 15. One rabbit given 0.5 μg of heparinase i.v. three times a week demonstrated antibodies by day 8. Five micrograms i.v. was consistently immunogenic when given on days 1 and 21, with antibody production on day 24. When 200 μg was given i.v. weekly, antibodies to heparinase were detected on day 7 in some cases and by day 15 in all rabbits.

Heparinase, 100 μg , was incubated with 100 μl of high-titer antiheparinase serum for 4 hr at room temperature. This was added to 1 ml of pooled rabbit plasma containing 4 U/ml heparin and incubated at 30° C for 1 hr, and the APTT was then determined. There was no loss in heparin-removing activity detected by either the APTT or azure A assays (Fig. 5). In a similar experiment, heparinase from a different fermentation in concentrations of 0 to 500 $\mu\text{g}/100 \mu\text{l}$ was incubated with 100 μl of high-titer antiheparinase serum or serum without antiheparinase antibody at room temperature for 4 hr. This 200 μl was then incubated with 1 ml of normal saline containing 10 U/ml heparin for 1 hr at 30° C. The azure A assay was then performed. No inactivation of enzyme by antiserum was demonstrable. When rabbits with high titers of antiheparinase antibodies were given i.v. heparinase, there was persistent heparin-removing activity (Fig. 6). The APTT after heparinase is significantly lower ($p < 0.05$) than without heparinase except at 45 min. On

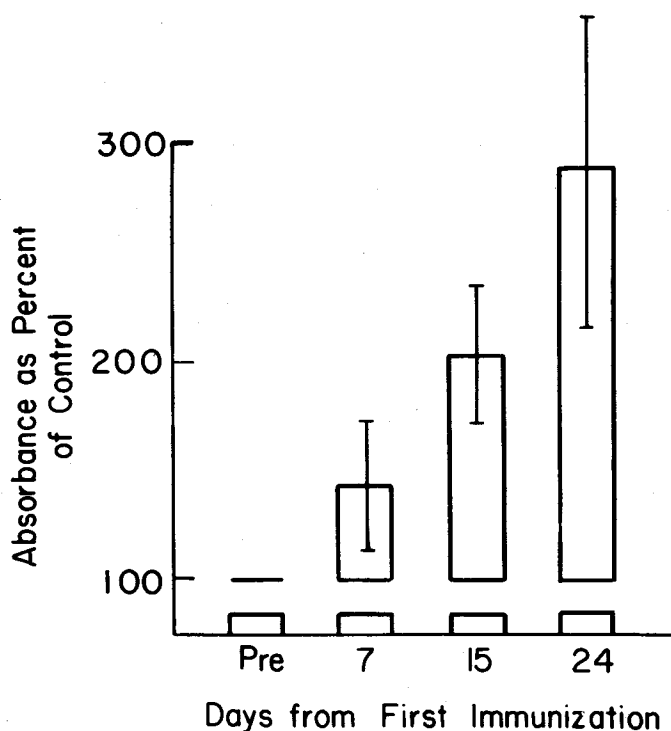


Fig. 4. Immunogenicity of heparinase in rabbits determined with the ELISA technique. Absorbance as a percent of preimmunization control serum is shown for five rabbits immunized i.v. or i.m. with Freund's adjuvant. Means \pm S.D. are displayed. Dose of heparinase varied from 100 to 200 μ g and number of challenges varied from two to nine.

the day of the experiment, these rabbits had ELISA absorbances three to four times control values, indicating that a high titer of antiheparinase antibodies was still present.

The complete blood count values in three rabbits receiving heparinase were averaged and compared to those in three similar rabbits anesthetized and bled in a similar fashion after receiving heparin but no heparinase. The hemocrit was 31.4 vol% in the heparinase rabbits and 30.6 in the controls. The hemoglobin was 10.4 gm/100 ml in the experimental rabbits (10.1 in controls); the white blood cell count was 4200/mm³ (3900/mm³ in controls); and the platelet count was 412,000 in the heparinase rabbits (433,000 in controls). Blood urea nitrogen, sodium, potassium, chloride, carbon dioxide, creatinine, and bilirubin (total and direct) did not differ from control values and neither did the SGOT, SGPT, LDH, alkaline phosphatase, amylase, and pancreatic lipase. No pathologic abnormalities of the tissues examined microscopically were noted.

Discussion

Heparinase was first isolated from bacteria by Payza and Korn¹¹ in 1956 in order to investigate the structure and nature of heparin. Since that time the production and isolation of heparinase from *Flavobacterium heparinum* has been investigated by several groups.^{6, 7, 12, 13} It appears to act in a random endolytic manner, cleaving the alpha glycosidic linkages in heparin¹⁴ and producing saccharide fragments with little anticoagulant activity.¹⁵

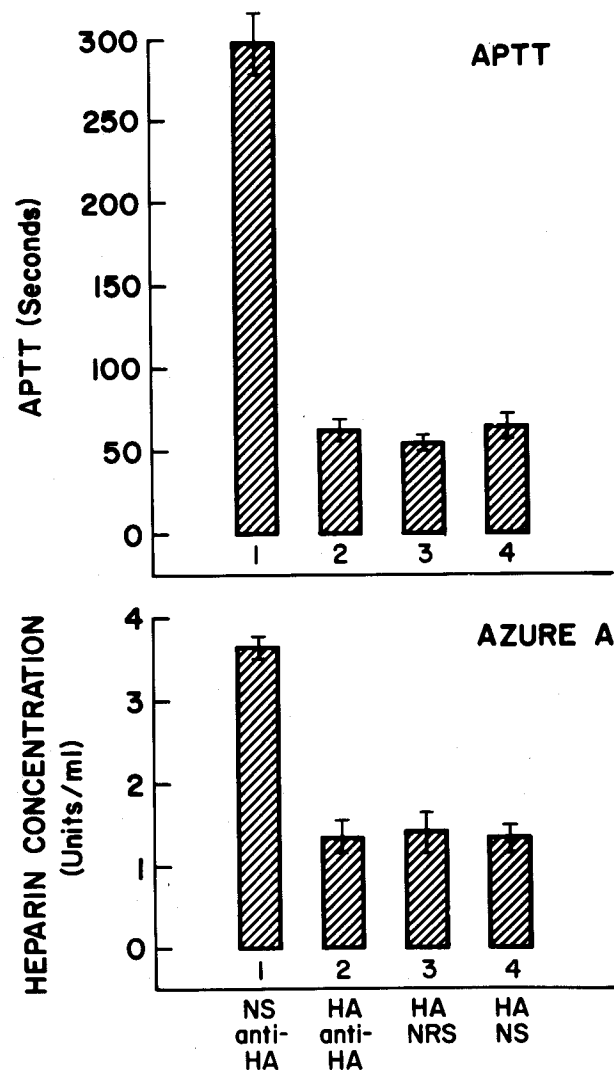


Fig. 5. In vitro activity of heparinase after incubation with high-titer antiheparinase serum. Total volume of 200 μ l each of the four groups was incubated 4 hr at room temperature. One milliliter of plasma with 4 U/ml heparin was added to the mixture and incubated at 30° C for 1 hr. Heparin levels were then determined with the APTT and azure A assays. Group 1, Normal saline (NS) incubated with high titer antiheparinase serum (*anti-HA*). Group 2, 100 μ g of heparinase (HA) incubated with high-titer antiheparinase serum. Group 3, 100 μ g of heparinase incubated with normal rabbit serum (NRS). Group 4, 100 μ g of heparinase incubated with normal saline.

In the experiments reported here, bacterial-derived heparinase degrades the anticoagulant activity of heparin in vitro in rabbit plasma and in vivo in rabbit blood. Parallel studies using the azure A assay demonstrate that chemical heparin is also degraded. Not all the anticoagulant activity of heparin is destroyed by heparinase. This is probably due to the anticoagulant activity of the remaining small fragments of heparin. The action of heparinase on heparin results in fragments of two to eight saccharide units,¹⁴ and certain

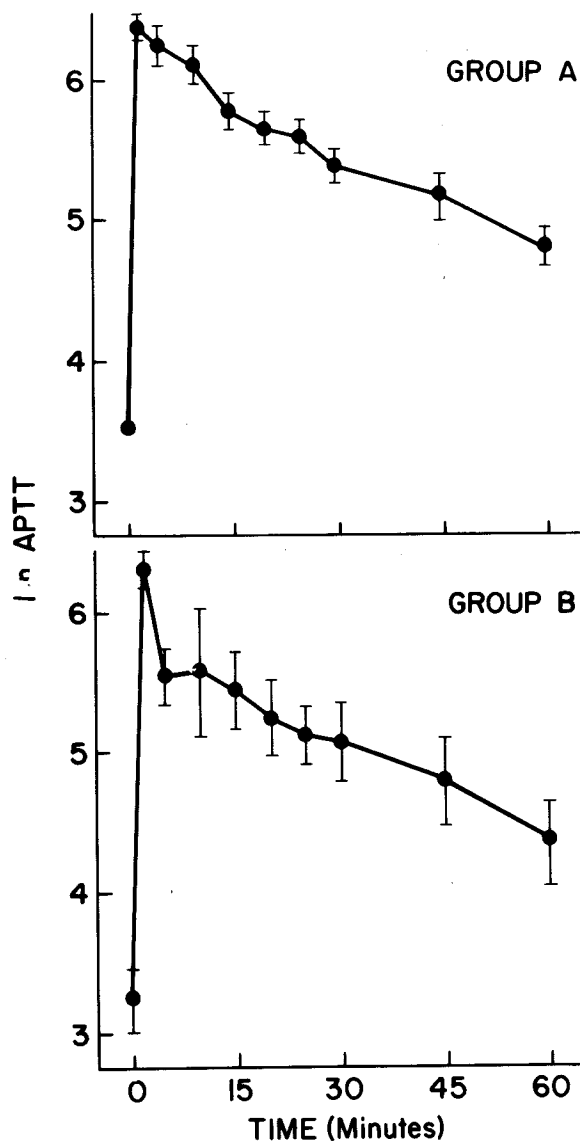


Fig. 6. In vivo activity of heparinase in rabbits with antiheparinase antibodies (mean \pm S.D., $n = 5$). Group A, 100 U/kg heparin given i.v. at 0 min. Group B, 100 U/kg heparin give i.v. at 0 min followed by 25 μ g/kg heparinase at 2 min.

of these fragments have some anticoagulant activity, although completely degraded heparin has no APTT activity. It may also be that some heparin is bound to endothelial cells¹⁶ and is released only after circulating heparin has been destroyed by heparinase, by which time the heparinase has been destroyed by some in vivo mechanism, perhaps nonspecific proteases. The fact that heparinase given prior to heparin administration is less active in degrading heparin than heparinase given after heparin administration indicates that the enzyme is either degraded in vivo or removed from circulation.

From a consideration of the in vivo heparin disappearance curves with and without

heparinase, it also appears that heparinase is quickly inactivated in the blood. Although the differences in heparin effect in vivo are statistically significantly different up to 20 min after heparinase administration, the difference attributable to heparinase itself is probably limited to the first 10 min. In a separate statistical treatment we used a two-sample univariate t test to compare the ratio of the maximum anticoagulant activity by APTT (2 min after administration of 100 U/kg heparin) to the anticoagulant activity at intervals after administration of heparinase or normal saline (the data presented in Fig. 2, *Groups A and B*). There was significantly less anticoagulant activity 5 and 10 min after heparin administration when heparinase was given.

We propose that heparinase activity is present early and reduces the initially large concentration of heparin. There is then the effect of an initially smaller dose of heparin. Lower doses of heparin have a shorter half-life than higher doses¹⁷ and thus the later part of the disappearance curves may only appear to be showing some continuing activity of heparinase but actually be demonstrating the shorter half-life of the lower initial heparin concentration. Therefore immobilized heparinase may be more effective than soluble heparinase because it may be more stable in blood.

Although the heparinase preparation used in this study is antigenic in rabbits, it appears that this antiheparinase antibody is not directed to the active site of the enzyme. The antibody might also be one produced in response to a contaminating protein. Although the answer to this question awaits the further purification of heparinase, the clinically important aspect, that the antibody does not affect the activity of the enzyme, is well supported here.

Heparinase had no specific toxic effects in these short-term experiments. Any differences in blood values from published normals observed in the study are explained by the multiple blood sampling employed in the experimental design.

Heparinase holds promise as a clinical heparin antagonist for many reasons. It destroys heparin rather than reversibly binding it and thus catalytic rather than stoichiometric doses might be used, and rebound effects such as those seen with protamine may not occur. Heparinase is highly specific and does not appear to participate in other chemical reactions, acting specifically on heparin. Therefore it may not cause the hypotension and anticoagulant effects that protamine causes.

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REFERENCES

1. Jastrzebski J, Sykes MK, and Woods DG: Cardiorespiratory effects of protamine after cardiopulmonary bypass in man. *Thorax* **29**:534, 1974.
2. Jaques LB: Protamine—antagonist to heparin. *Can Med Assoc J* **108**:1291, 1973.
3. Hampers CL, Blafox MD, and Merrill JP: Anticoagulation rebound after hemodialysis. *N Engl J Med* **275**:776, 1966.
4. Langer R, Linhardt RJ, Galliher PM, Flanagan MM, Cooney CL, and Klein MD: A system for heparin removal. In: *Biomaterials: Interfacial Phenomena and Applications*, Cooper SL, Peppas NS, Hoffman AS, and Rather BD, editors. Washington D.C., 1982, American Chemical Society, *Advances in Chemistry Series*, vol. 199, p. 493.
5. Langer R, Linhardt RJ, Hoffman S, Larson AK, Cooney CL, Tapper D, and Klein M: An enzymatic system for removing heparin in extracorporeal therapy. *Science* **217**:261, 1982.
6. Galliher PM, Cooney CL, Langer R, and Linhardt RJ: Heparinase production by *Flavobacterium heparinum*. *Appl Environ Microbiol* **41**:360, 1981.

7. Galliher PM, Linhardt RJ, Conway LJ, Langer R, and Cooney CL: Regulation of heparinase synthesis in *Flavobacterium heparinum*. Eur J Appl Microbiol Biotechnol **15**:252, 1982.
8. Bjornsson TD and Wolfram KM: Determinants of the anticoagulant effect of heparin *in vitro*. Ann NY Acad Sci **370**:656, 1981.
9. Klein MD, Drongowski RA, Linhardt RJ, and Langer RS: A colorimetric assay for chemical heparin in plasma. Anal Biochem **124**:59, 1982.
10. Voller A, Bidwell D, and Bartlett A: Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In: Manual of Clinical Immunology, Rose NR and Friedman H, editors. Washington D.C., 1976, American Society for Microbiology, p. 506.
11. Payza AN and Korn ED: Bacterial degradation of heparin. Nature **177**:88, 1956.
12. Hovingh P and Linker A: The enzymatic degradation of heparin and heparitin sulfate. III. Purification of a heparitinase and a heparinase from Flavobacteria. J Biol Chem **245**:6170, 1970.
13. Silva ME and Dietrich CP: Structure of heparin: characterization of the products formed from heparin by the action of a heparinase and a heparitinase from *Flavobacterium heparinum*. J Biol Chem **250**:6841, 1975.
14. Linhardt RJ, Fitzgerald GL, Cooney CL, and Langer R: Mode of action of heparin lyase on heparin. Biochim Biophys Acta **702**:197, 1982.
15. Linhardt RJ, Grant A, Cooney CL, and Langer R: Differential anticoagulant activity of heparin fragments prepared using microbial heparinase. J Biol Chem **257**:7310, 1982.
16. Jaques LB: Heparin: an old drug with a new paradigm. Science **206**:528, 1979.
17. McAvoy TJ: Pharmacokinetic modeling of heparin and its clinical implications, J Pharmacokinetic Biopharmacol **7**:331, 1979.