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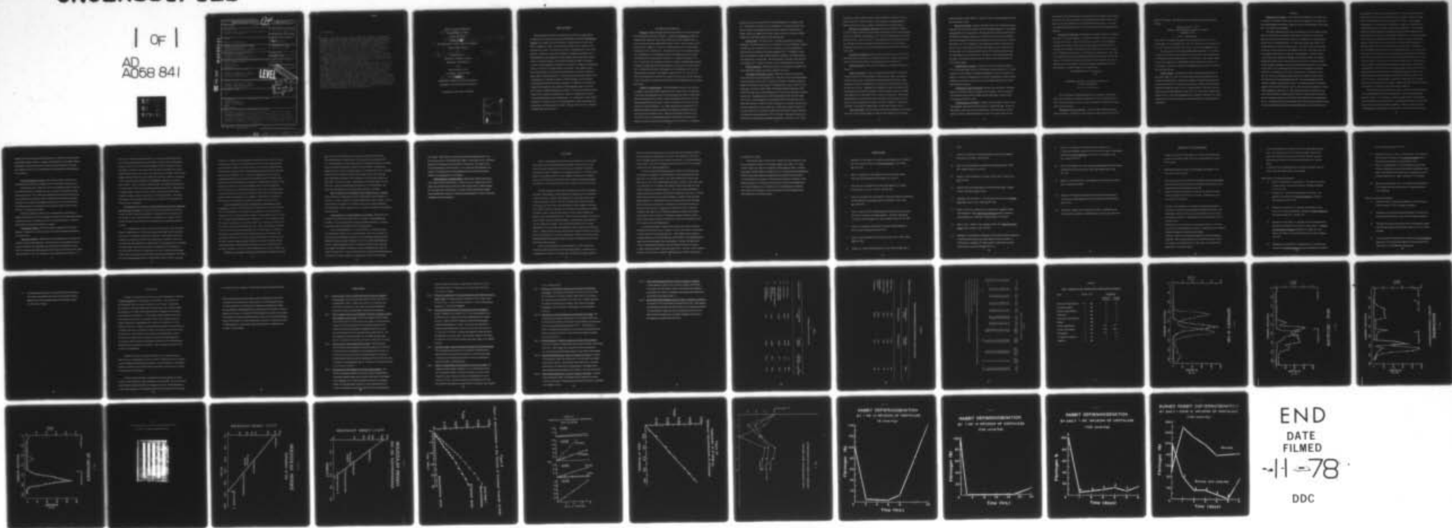

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Crotalase, the thrombin-like enzyme from Eastern diamondback rattlesnake (*Crotalus adamanteus*), has been purified to homogeneity. This was shown by chromatographic and electrophoretic patterns of the enzyme. A single band was observed on SDS polyacrylamide gel electrophoresis. The molecular weight was estimated to be about 33,000 by gel filtration on Sephadex G-100 and SDS polyacrylamide gel electrophoresis. The molecular weight determined

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from amino acid composition was shown to be 30,334. However, the estimated molecular weight contributed by carbohydrates (2013) gave us an overall value of 32,347. Analytical isoelectric focusing of crotalase showed five bands. This heterogeneity of enzyme may be due to differences in sialic acid content of the different forms of the enzyme. Upon treatment of the enzyme with neuraminidase to remove the sialic acid a single band was obtained by isoelectric focusing, supporting our contention that the enzyme is homogeneous and that the five bands represent different levels of sialylation of the enzyme. This heterogeneity may have been an artifact of preparation.

Fibrinolytic activity of crotalase was shown to be an inherent property since the ratio of fibrinogenolytic activity to that of fibrinolytic activity remained constant throughout the purification procedure. Crotalase hydrolysed a chromogenic substrate (S-2238) used for thrombin assay; this activity could be used as a method for estimation of crotalase.

We have utilized crotalase to investigate the possible application of this enzyme in clinical situations using an experimental animal system. We have developed a model system utilizing the rabbit to investigate the effect of defibrinogenation on burn injury and repair. Dose response studies have been performed to determine the optimum level of crotalase to defibrinogenate rabbits during burn trauma. Control studies have shown that plasma levels of fibrinogen rise from 100% in normal to 250% following 5% total body burn. In non-traumatized rabbits intravenous infusion of 4-6 units NIH units of crotalase per kilogram body weight for 1 hour produce a defibrinogenated state which lasts for 6-12 hours. In burned animals, daily infusion of crotalase (50-100 units/kg body weight) has successfully been employed to maintain defibrinogenation in spite of the increased fibrinogen levels following burns. In further experiments we propose to evaluate the effect of defibrinogenation on burn repair using revascularization in burned area as an index of wound healing.

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FINAL REPORT, 1 Jul 76-1 Jul 77,

The Application of a Snake Venom Anticoagulant Enzyme in Burn Therapy.

by

Francis S. Markland, Jr

University of Southern California

School of Medicine

Department of Biochemistry

and

Cancer Research Center

Los Angeles, California 90033

30 June 1978

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ANNUAL REPORT

This final report deals with our research from 1 July 1976 to 1 July 1978 on the purification of crotalase from eastern diamondback rattlesnake (Crotalus adamanteus) and its application in burn therapy. During this period we have been able to standardize a large scale purification procedure for the isolation of this thrombin-like enzyme. The enzyme was shown to be homogeneous on SDS electrophoresis. In addition, we have used crotalase to investigate the possible role of defibrinogenation (anticoagulation) in burn therapy using an experimental animal system. We have developed a model system utilizing the rabbit to investigate the effect of anticoagulation as induced by crotalase for comparison with published work on the efficacy of heparin therapy in burn injury. Dose response studies have been performed to determine the optimum level of crotalase to defibrinogenate rabbits during burn trauma. Control studies have shown that plasma levels of fibrinogen rise from 100% in normal to 250% following 5% total body burn. In nontraumatized rabbits intravenous infusion of 4-6 NIH units of crotalase per kilogram body weight per hour produced a defibrinogenated state which lasted for 6-12 hours. In burned animals, daily infusion of crotalase (50-100 units/kg body weight) has successfully been employed to maintain defibringenation in spite of the increased fibrinogen levels following burn. As a continuation of these studies, we propose to evaluate the effect of defibrinogenation on burn repair using revascularization in the burned area as an index of healing. This work will be supported by USPHS, NIH Grant HL 22875.

MATERIALS AND METHODS

Materials: Human fibrinogen and N-acetyl neuraminic acid were purchased from Calbiochem, San Diego, California. Lyophilized C.Adamanteus venom was obtained either from Ross Allen Venom Laboratories, Silver Springs, Florida, or more recently from Miami Serpentarium, Miami, Florida. DEAE-cellulose (DE-52) was from Reeve Angel Co., Clifton, N.J. Neuraminidase was from Worthington Biochemicals Corp., Freehold, N.J. Calibration kit for molecule weight determination, SP-Sephadex (C-50), Sephadex G-100 and CH-Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Standard thrombin (Lot No. B-3, 21.7 units/mg) was obtained from Division of Biologic Standards NIH, Bethesda, Maryland. Benzamindine hydrochloride hydrate was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. The reagents for electrophoresis were from Bio-Rad, Richmond, California. Chromogenic substrates S2251, H-D-Val-Leu-Lys-pNA; S-2222, BZ-Ileu-Glue-(γ-OR)-Gly-Arg-pNA; S-2238, H-D-Phe-Pip-Arg-pNA; S-2160, N-Bz-Phe-Val-Arg-pNA were obtained from Orthodiagnostics Inc., Raritan, New Jersey.

Methods: Chromatography: All chromatographic materials were treated according to the manufacturer's requirements; in all cases resins were suspended in the initial chromatography buffer with decanting several times to remove fines. The initial eluting buffer was used to wash newly prepared columns for 24 hours before applying the protein sample. Protein concentration in fractions was measured by the 280 nm absorbance. All column operations were performed at 4°C. Benzamidine affinity martrix was prepared by washing CH-Sepharose 4B (15 g) with 0.5 M NaCl and distilled water. The gel was suspended in distilled water (60 ml) and 1-ethyl-3(3-dimethyl aminopropyl)-carbodiimide (3.0g) was slowly added in 15 minutes while pH was maintained at 4.75 with 1N HCl. p-Aminobenza-

midine (0.6 g in 4.0 ml water, pH 4.75) was added dropwise in 20 minutes at pH 4.75. The mixture was stirred at room temperature for 5 hours at pH 4.75. The gel was washed with distilled water and the above steps were repeated twice. Finally, the gel was washed with 2N NaCl, water, 1M acetic acid, water, 0.001M NaOH, water and 0.05M Tris-HCl-0.15M NaCl (pH 9.0).

Enzyme Assay: Clotting activity of crotalase was assayed with human fibrinogen. The venom solution (0.1 ml) was added to 0.2 ml of fibrinogen (10 mg/ml in 0.04M Tris-HCl, 0.14M NaCl, pH 7.35). The time for the first appearance of fibrin strands was measured with gradual shaking at 37°C. A standard clotting curve was obtained by adding varying amounts of thrombin to fibrinogen solution and measuring the clotting times. The clotting times of crotalase were used with this standard curve to obtain the venom activity in NIH thrombin clotting units.

Protein Estimation: Protein concentration of crotalase was obtained from 280 nm absorbance using an extinction coefficient of 14.8 (1).

Screening of Fibrinolytic Activity: Fibrinolytic activity was estimated by the fibrin plate assay using plasmin (10.7 caseinolytic units/ml, Michigan Bureau of Public Health, courtesy of Dr. H. Sqouris) as standard. Fibin plates were prepared by the method of Kluft et al. (2). Fibrinogen stock solution 1.65% in 0.07M $(NH_4)_2SO_4$ was diuted in plate buffer (0.05M sodium diethyl barbiturate, 0.093 M NaCl, 1.66 mM $CaCl_2$, 0.69 mM $MgCl_2$, pH 7.8) to give final fibrinogen concentration of 0.1%. Fibrinogen solution (6 ml) was then pipetted into flat petri dishes (90 x 15 mm). After mixing with 0.2 ml thrombin solution (20 NIH units/ml) the dishes were allowed to stand for at least 30 minutes on a carefully levelled surface at room temperature to form a fibrin layer. Small holes of specific diameter (2 mm) were made at 1.5 cm intervals in fibrin layer and 0.005 ml sample was added to different holes and incubated at 37°C for 18 hours. Fibrin plate clearance was determined by measuring two perpendicular diameters of the lysed zone. Incuba-

tions were in triplicate and the mean of the six diameters was taken as the diameter of the zone of fibrin clearance. Zone clearance of standard plasmin at different dilutions was used to prepare a standard curve of fibrin clearance.

Hydrolysis of Chromogenic Substrates: The reaction mixture consisting of 1.1 ml of buffer (0.05 M Tris-0.125 M NaCl-0.025 M HCl, pH 8.3), 0.05 ml enzyme and 0.1 ml of substrate was incubated at 37°C for 3 min and the reaction was stopped with 0.05 ml of 50% acetic acid. p-Nitroaniline yellow color was read at 405 nm. Thrombin was used as standard with substrate S-2238. Since crotalase is a thrombin-like enzyme, most of the experiments presented here were conducted with S-2238.

Disc gel electrophoresis: This procedure was carried out at 4°C with 7.5% polyacrylamide gel at pH 8.3, by the method of Davis (3). The gels were stained with Coomassie Brilliant Blue R-250 and washed with 7.5% acetic acid.

SDS Electrophoresis: This was run according to the method of Weber and Osborn (4).

Amino acid analysis: Amino acid analysis was performed by the method of Moore and Stein (5) with the Durrum D-500 automatic amino acid analyser. Salt-free venom enzyme was prepared by dialysing exhaustively against water and duplicate aliquots containing 50 ug of protein were hydrolysed for 24 hours, 48 hours, and 72 hours. Tryptophan was determined by amino acid analysis after hydrolyzing 180 ug of protein in duplicate with 4N methane sulfonic acid reagent containing 0.2% 3-(2-aminoethyl)-indole at 115°C for 20 hours (6). Half-cystine was determined as cysteic acid by amino acid analysis after performic acid oxidation (7) and acid hydrolysis of 330 ug of crotalase in duplicate. Cysteine was estimated by the method of Ellman using 5,5-dithio-bis-2-benzoic acid (8).

Molecular weight: This was estimated by SDS electrophoresis (4) and Sephadex G-100 column chromatography (9) using the following proteins as molecular

weight standards: adolase, 158,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; and ribonuclease, 13,900.

Isoelectric Focusing: Analytical thin-layer isoelectric focusing on polyacrylamide gel was conducted by using the LKB 2117 Multiphor apparatus. 30 ug samples were applied by use of a paper strip. The solutions of the cathode and anode were 1M NaOH and 1M H_3PO_4, respectively. Initially, 150 volts (65 mA) were applied which was increased by 10 volts each 10 minutes until the current became constant after about 2 hr. Upon completion of the experiment (2 hours), the pH was measured at each 0.5 cm distance and the pH versus distance (cm) was plotted. The gel was stained with Coomassie Brilliant Blue R-250 in methanol and destained in water:ethanol:acetic acid (8:3:1 v/v). The distance of the protein bands from the origin were measured and the isoelectric points were determined from the pH vs distance graph.

Neuraminidase Treatment: Crotalase (5 mg) was incubated overnight at 37°C with neuraminidase (5 units) in 0.1M sodium acetate buffer (pH 5.0) in a final volume of 1 ml and samples containing the appropriate amount of crotalase were applied to isoelectric focussing plates or disc gel electrophoresis. Blanks of neuraminidase and crotalase at zero time or after overnight incubation were also run under identical conditions.

Carbohydrate Analytical Methods: Hexoses were estimated by the phenol-sulfuric acid method of Hirs (10) using glucose as standard. Sialic acid content was estimated by the method of Warren (11) after hydrolysis of venom enzyme in 0.1N H_2SO_4 at 80°C for 1 hour.

Defibrinogenation of Rabbits: A rabbit of known weight was kept in a specially designed cage which allowed the head to be outside the cage. The hair on the outside of one of the ears was removed with a razor blade and the ear was taped on a metallic illuminated platform in front of the cage so that the veins

were exposed. The venom enzyme (of known units/kg body weight) in sterile saline was injected into the ear vein and 1 ml blood samples were removed and the fibrinogen content was estimated at two hour intervals after injection. A zero time blood sample was withdrawn prior to venom enzyme infusion for fibrinogen determination.

Estimation of Fibrinogen: Fibrinogen was estimated by using 0.9 ml venous blood with 0.1 ml of anticoagulant 1:1 mixture of trisodium citrate (32.0 g/liter of saline) and ε-amino- caproic acid (100 g/liter in saline) and centrifuged at 1,000 rpm for 20 minutes. Then 0.25 ml of the plasma was mixed with 2.75 ml of buffer (0.10 M veronal HCl 0.04M-NaCl, pH 7.2) and 0.025 ml of Ca^{++}-thrombin (2.5 NIH units in 0.025M $CaCl_2$). This mixture was incubated for 1 hour. The clot formed was removed with a glass rod and washed with normal saline and then water for 30 minutes each and dissolved during a 2 hour treatment with 5 ml of alkaline urea. The abosorbance was then read against alkaline-urea at 282 nm and fibrinogen is estimated using the following formula:

$$\text{mg fibrinogen/ml blood} = \frac{\text{O.D.} - 0.01}{1.617} = X$$

$$\text{mg fibrinogen/ml blood} = X \times \text{dilution of plasma} \times \text{vol. of urea} \times \text{blood dilution}$$

$$= X \times 2 \times 5 \times 1.1 \text{ (in this case)}$$

The percentage of fibrinogen remaining in the plasma at various times after venom infusion is calculated by taking the zero time of fibrinogen level as 100%. Bovine fibrinogen was used to obtain a standard curve using the same procedure as described above.

Preparation of Mold for Burning: A mold for rabbits during third degree burn was prepared. All rabbits in these studies have approximately the same body

weight (8-8.5 pounds). The surface area was calculated by using the following formula:

$$\text{Surface Area } (cm^2) = kw^{2/3}$$

where k = a proportionality constant for the rabbit (assumed to be 10)

and w = weight in grams.

For a rabbit of 8.5 pounds, the surface area calculated was 2,495 cm^2. So for 5% body burn: 5% of this surface area = 123 cm^2. Thus a hole with a surface area of 123 cm^2 was cut in the middle of the mold. This hole is rectangular in shape.

The mold is about 55 cm long (assuming rabbit dimensions of 30 cm trunk, 5 cm neck, 10 cm head = 45 and leaving 5 cm on each side so that the animal may be comfortable) and the width is about 10 cm. The mold is a half cylinder (length 55 cm x 20 cm diameter) cut longitudinally and as indicated has a hole 123 cm^3 in the middle. This mold is covered with insulating material to protect the animal from additional burning.

Animal Burning: All rabbits used in these studies had approximately the same body weight (8-8.5 lbs). Total body surface area was calculated from the formula as described above. Rabbits were clipped of back hair and residual hair was removed with Neet, a commercial hair remover. Rabbits were placed under anesthesia (Nembutal sodium, 1.0 ml of 60 mg/ml solution). The animal was positioned in the mold and immersed in water at 75°C for 10 seconds to produce third degree scald (12). The water temperature was controlled to within 0.1°C by a temperature control device and water was continuously stirred to maintain uniform temperature.

RESULTS

Purification of Crotalase: A new method was developed for the purification of crotalase. The method consists of gel filtration on Sephadex G-100, DEAE cellulose chromatography, benzamidine-Sepharose affinity chromatography and SP-Sephadex-C-50 column chromatography.

The crude lyophilized venom (5.0 g) was dissolved in 20 ml of 0.04M Tris-HCl (pH 7.1)-0.1M NaCl and centrifuged at 5000 rpm for 10 minutes to remove small amounts of insoluble materials and loaded onto a Sephadex G-100 column preequilibrated with the solubilizing buffer. Two major and several minor peaks at 280 nm absorption were obtained as shown in Fig. 1. The active fractions were pooled, 26,250 units were recovered from the applied 28,000 units. This fraction was dialysed against 0.005M sodium acetate (pH 7.0) and applied to a DEAE-cellulose column, previously equilibrated overnight with the dialysis buffer. The dialysed sample was loaded onto the column and eluted with 100 ml of the dialysis buffer. A linear gradient consisting of 425 ml of starting buffer and 425 ml of 0.1M sodium acetate (pH 7.0) was initiated and followed with a second gradient established by 400 ml of 0.1M sodium acetate (pH 7.0) and 400 ml of 0.7M sodium acetate (pH 7.0). Fractions containing crotalase activity were pooled; overall recovery after DEAE-celllose chromatography was 92%. The chromatographic pattern of DEAE-cellulose column is shown in Fig. 2. The pooled fractions were dialysed against 0.05M Tris-HCl-0.15M NaCl, pH 9.0, and the sample was applied to a benzamidine-Sepharose column (Fig. 3). The column was eluted successively with 80 ml of 0.05 M Tris-HCl-0.15M NaCl, pH 9.0, 200 ml of 0.05M Tris-HCl-0.4M NaCl, pH 9.0, and, finally with 250 ml of 0.4M Tris-HCl-0.10M NaCl-0.15M benzamidine, pH 9.0. Crotalase eluted in the benzamidine wash and the fractions having crotalase activity were checked on analytical polyacrylamide disc gel electrophoresis after

dialysis against 0.05M Tris-glycine buffer (pH 8.9). The electrophoretic pattern showed that the first half of the peak (the left side in Fig. 3) showed two bands, whereas, the trailing half showed a single band. The affinity chromatography was repeated but it was observed to be difficult to separate crotalase from the contaiminating protein. However, the recovery was good (83%). This procedure showed that further purification required an additional chromatogrphy method and this problem was solved by employing SP-Sephadex (Fig. 4). The enzymatically active fractions from benzamidine-Sepharose column were pooled and dialysed against 0.005M sodium acetate, pH 5.5, buffer. Elution from SP-Sephadex C-50 employed initially 140 ml of 0.05M sodium acetate buffer, pH 5.5, followed by the first gradient which utilized 75 ml of starting buffer and 75 ml of 0.1M acetate buffer, pH 5.5, and finally by a second linear gradient utilizing 100 ml of 0.1M acetate buffer, pH 5.5, and finally by a second linear gradient utilizing 100 ml of 0.1M acetate buffer, pH 5.5, and 100 ml of 0.7M acetate buffer, pH 5.5. Purified crotalase was present in the second peak and showed a single band on acrylamide gel electrophoresis. These fractions were pooled and again checked by SDS electyrophoresis and enzyme was observed to be homogeneous as shown in Fig. 5. The pooled fractions were dialysed against 0.5M Tris-HCl-0.1M NaCl, pH 7.35, and tested for activity. This procedure of purification showed that 50% of enzyme activity was recovered with a purification of about 60-80 fold. The enzyme solution was divided in test tubes (1 ml each) and frozen for further use.

The overall scheme of purification is shown in Table I. The specific activity of the final preparation was 407 units/mg protein. Since crotalase has fibrinogenolytic activity, it was of interest to find out whether there is any fibrinolytic activity residing in crotalase molecules. To investigate fibrinolytic activity, fibrin plate method was used as described in Methods. As shown in Table II, the ratio of fibrinogenolytic and fibrinolytic activity (specific activity) remained constant

showing that both activities are inherent property of crotalase. Since this experiment requires further evidence to support this hypothesis, we will conduct further experiments involving modification of different functional groups of crotalase and compare the effect of the modifications on both fibrinolytic and fibrinogenolytic activiites.

Molecular Weight of Crotalase: The molecular weight of crotalase was determined by gel filtration on Sephadex G-100 (9) and SDS electrophoresis (4).

In gel filtration the column (90 x 1.25 cm) was equilibrated with 0.05M Tris-HCl-0.15M NaCl, pH 9.0, overnight and the void volume was estimated by using blue dextran (63 ml). The column was standardized with proteins of known molecular weight and the molecular weight of crotalase was estimated by applying crotalase to the column and measuring clotting activity in the eluted fractions. The elution pattern of venom enzyme and of marker proteins is shown in Fig. 6. The mol. wt. estimated was 33,000.

In SDS electrophoresis, the mol. wt. was determined in the presence of sodium dodecyl sulfate (4). The electrophoretic mobility of crotalase and of marker enzymes were used to calculate the mol. wt. of about 36,000 (Fig. 7). We have used 33,000 as the molecular weight for crotalase.

Carbohydrate analysis: The venom enzyme contained 6.1% total carbohydrates. In addition, the content of sialic acid varied in different preparations from 3.4 to 6.3%.

Amino Acid Analysis: Samples from several preparations of crotalase were checked for amino acid composition. The data for one of the preparations is presented in Table III. The composition was calculated after hydrolysis for 24,48 and 72 hr in duplicate by determining the molar ratios of each amino acid to alanine which was taken as 1.00. The average ratio was multiplied by the residue wt. of

amino acid to obtain the minimum residue wt. These were summed and divided into the physically determined molecular weight of 30987 (without carbohydrate). This gave a factor of 13.78, which when multiplied times the average ratio, gave the number of residues of each amino acid, this value was rounded off to nearest integral number which gave the amino acid composition of crotalase. The molecular weight determined from amino acid composition was 30334. However, the addition of the molecular weight contributed by carbohydrates (2013) gave us an overall value of 32347. Amino acid analysis after performic acid oxidation and hydrolysis of crotalase gave 20 half cystine residues indicating the presence of approximately 10 disulfide bridges. There was no free cysteine as determined by the method of Ellman (8) using crotalase in the native or in the denatured state. The values differ slightly from those previously reported for crotalase (1), but in general the results are quite comparable.

Electrophoretic and isoelectric focussing profiles before and after neuraminidase treatment of crotalase. Generally on disc gel electrophoresis of crotalase, we have observed a single broad band after Coomassie Blue G-250 staining. We felt this may be due to different amounts of carbohydrate in crotalase. Recent reports with ancrod and batroxobin (13-15) prompted us to analyze this situation in detail.

In our experiments, crotalase (15 ug) showed three bands on disc gel electrophoresis (a larger amount of enzyme gives single broad band) but after treatment with 5 units of neuraminidase and reelectrophoresis a single band was observed. Similarly, we checked the isoelectric focusing profile in crotalase. In this case, we found three major bands and two minor bands. In some preparations, we obtained five major bands. The variation may be due to different levels of sialic acid. It is also possible that native crotalase has a finite level of sialylation but during isolation part of the sialic acid is hydrolysed, especially during dialysis or SP-Sephadex column chromatography where acidic pH buffer is used. However, after the

treatment of crotalase with neuraminidase and reanalysis on isoelectric focusing a single band was observed. One problem was encountered since neuraminidase and desialylated crotalase focus at the same pH. We, therefore, separated them by benzamidine-Sepharose affinity chromatography and desalted the pooled cortalase fractions on Sephadex G-25. Crotalase was again analyzed by isoelectric focusing and virtually the same isoelectric focussing profile was observed as described above following neuraminidase treatment; a single band was obtained demonstrating the homogeneity of the neuraminidase treated crotalase preparation. The isoelectric point of this band was found to be 4.60. Hence, crotalase, similar to batroxobin and ancrod, has an acidic isoelectric point; the microheterogeneity probably represents varying levels of sialylation of the protein. This indicates that the protein is homogeneous except for the variable sialic acid content.

Action of neuraminidase on the fibrinogenolytic and amidolytic activity of crotalase: In view of the heterogeneity in crotalase due to sialic acid, and the effect of neuraminidase on removing this heterogeneity, we were interested in studying the effect of neuraminidase on amidolytic and coagulant activity of crotalase. Crotalase and neuraminidase were incubated at 37°C and coagulant activity was observed at different time intervals. There was no change in clotting activity of crotalase following neuraminidase treatment. When the chromogenic substrate S-2238 was incubated with neuraminidase alone, crotalase alone, or crotalase in the presence of neuraminidase and the hydrolysis product (p-nitroaniline) was estimated at different time intervals by spectrophotometeric observations at 405 nm (Fig. 8) there was slight increase in amidolytic activity of crotalase treated with neuraminidase. Neuraminidase alone had no action on S-2238. Hence the increase in amidolytic acitvity of crotalase might be due to removal of sialic acid.

Hydrolysis of chromogenic substrates with different concentrations of crota-

lase: Different concentrations of crotalase were incubated with different chromogenic substrates and liberation of p-nitroaniline was monitored at 405 nm. The results shown in Fig. 9, show that S-2160 is highly susceptible to crotalase whereas crotalase hydrolyses S-2251 and S-2222 very slowly. Crotalase was slightly less active with the thrombin substrate S-2238 than with S-2160. Thrombin showed about two times more activity than crotalase with S-2238 under the same experimental conditions. Since crotalase is a thrombin-like enzyme, we have used S- 2238 for our studies. Additionally, we have shown (Fig. 10.), that there was a linear relationship between hydrolysis of S-2238 substrate. This shows that this substrate can be used for the estimation of crotalase.

Effect of inhibitors on the amidolytic and clotting actitivy of crotalase: Several inhibitors of serine proteases were investigated for their inhibition of crotalase. As shown in Table IV no trypsin inhibitor inactivated crotalase. m-Aminobenzamidine was a highly active inhibitor followed by p-aminobenzamidine and benzamidine. Other compounds such as L- arginine-L-aspartate and agmatine were ineffective.

Defibrinogenation of Normal Rabbits with Crotalase. Experiments were performed to determine the optimal dose of crotalase for defibrinogenation of rabbits. This was accomplished by injecting different amounts of crotalase per kg body weight in the rabbits used for the experiment.

In the initial experiments, 1.2, 2.4 and 4.8 units of crotalase per kig of body weight were infused over a 5 min time period. There was a constant but small decrease in fibrinogen content up to 2-4 hr, whereupon, fibrinogen levels returned to normal by about 6 hrs (Fig. 11). In other words, defibrinogenation was not complete and lasted only for a few hours. However, when 6 units/kg body weight was infused over a period of 1 hour, defibrinogenation lasted for about 6-8 hours (Fig. 12). Since our objective was to keep rabbits defibrinogenated for periods of one day or longer, we tried infusion of 40, 60, or 100 units/kg

body weight. These levels of enzyme kept rabbits defibrinogenated for 18-24 hr as shown in Fig. 13 (100 units/kg body weight). To prolong the time of defibrinogenation, 100 units/kg was injected daily for several days. The result of one of those experiments is shown in Fig. 14. It is clear from this experiment that rabbits can be maintained in a fibrinogenated state for several days by daily infusion of the appropriate level of enzyme.

Defibrinogenation of Burned Rabbits: Similar dose response experiments were conducted with burned rabbits. As shown in Fig. 15, plasma levels of fibrinogen (an acute phase protein) rose from 100% (normal level) to 250% following 5% total body burn. Daily infusion of crotalase (50-100 units/kg body weight) has successfully been employed to maintain defibrinogenation, in spite of increased fibrinogen levels following burn.

DISCUSSION

The new chromatographic procedure for the purification of the thrombin-like enzyme from eastern diamondback rattlesnake is unique in that we can obtain enzyme having high specific activity in good yield and in a relatively short period of time as compared to our original procedure (1). The major characteristic of this method is that it does not require concentration of enzyme at any step. We found that concentration of the crotalase by ammonium sulphate fractionation or Amicon ultrafiltration leads to partial loss of enzyme activity.

The final preparation always showed homogeneity on SDS electrophoresis and other chromatographic methods based on size separation such as Sephadex G-100. The enzyme contained carbohydrates and is thus a glycoprotein with a molecular weight of 33,000. The other thrombin-like enzymes from snake venoms have also been shown to generally have molecular weights in the range from 29,100 to 35,400 (16). The amino acid composition of thrombin-like enzymes are known to have a relatively high content of acidic residues (aspartic and glutamic acid), relatively high content of proline and large amounts of half cystine, most of which is present in disulfide bridges. A similar amino acid composition was also observed in crotalase. Since ancrod and batroxobin (13-15) contain high amounts of acidic residues, this probably account for their acidic isoelectric points. A similar situation was also expected with crotalase; indeed the isoelectric point was determined to be 4.6 after desialylation. However, the enzyme without disialylation showed three major bands on isoelctric focusing profile all of which had acidic isoelectric points.

Since crotalase hydrolysed thrombin substrate S-2238, crotalase can be estimated spectrophetomericially by using this substrate. Amidolytic and fibrinogenolytic acitivites of crotalase were not inhibited by trypsin inhibitors.

However, p-aminobenzamidine, m-aminobenzamidine and benzamidine inhibited both fibrinogenolytic and amidolytic activities. The estimation of fibrinolytic activity throughout purification procedures showed that it is an inherent property of this enzyme. The ratio of fibrinogenolytic and fibrinolytic acitvity remained constant after each chromatograph step.

We have observed the defibrinogenation of rabbits with different doses of crotalase to investigate its clinical potential. The results presented in this report show that crotalase, similar to the other thrombin-like enzymes from snake venoms, can act as a defibrinogenating agent in animals. In previous studies in our laboratory (17) it was shown that infusion of purified crotalase into dogs caused a benign state of defibrinogenation. There was no fibrin deposition in internal organs and disseminated intravascular coagulation did not occur. Clotting studies showed that there were minimal changes in coagulation factors during defibrinogenation. It would appear that this venom enzyme attacks fibrinogen directly and removes this protein by formation of soluble fibrin monomers or abnormal fibrin microclots. Apparently the fibrinolytic system is activated secondarily since high levels of fibrinogen degradation products are produced concomitantly with the decrease in fibrinogen. Separate studies have shown that crotalase neither activates the fibrinolytic system nor is fibrinolytic itself at the levels used for defibrinogenation.

In certain pathological conditions such as burn injury, tumor metatasis, thrombosis and infection, there is evidence for a direct involvement of fibrinogen (or fibrin deposition) in the pathologic state. Anticoagulants such as heparin, coumarin derivatives, factor XIII inhibitors, and fibrinolytic and defibrinogenating agents have been used against these abnormalities. Recently, the application of venom defibrinogenating agents such as Batroxobin and Ancrod in certain of these conditions has given interesting results (18-21). However, in some instances animals may not have been completely defibrinogenated, thereby complicating

interpretation of results.

In the present study, we have tried to evaluate the effectiveness of crotalase as compared to heparin as a therapeutic agent in burn repair. In so doing we have insured that animals are completely defibrinogenated prior to studying burn repair. Further studies to evaluate the role of (crotalase induced) defibrinogenation on burn repair using revascularization in the burned areas as an index of wound healing are in progress. These results should help to determine if fibrin deposition plays a role in burn injury and may also establish if defibringenation is an effective therapy for this condition or for other pathological conditions such as deep vein thrombosis or tumor metastasis where fibrin deposition has been implicated in the disease process.

BIBLIOGRAPHY

1. Markland, F.S. and Damus, P.S. Purification and Properties of a Thrombin-like enzyme from the venom of Crotalus adamenteus. J. Biol. Chem. 246: 6460, 1971.

2. Kluft, C., Brankman, P. and Veldhuyzen-Stolk, E.L.; In Prog. Chem. Fibrinolysis and Thrombolysis (Ed. Davidson) N.Y. 57, 1976.

3. Davis, B.J. Disc electrophoresis II, Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121: 404, 1964.

4. Weber, K. and Osborn, M. The reliability of molecular weight determinations by dodecylsulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406, 1969.

5. Moore, S. and Stein, W.H. Chromatographic determination of amino acids by the use of automatic recording equipment. Methods of Enzymology 6 (Colowick, S.P. and Kaplan, N.O., Eds.) Academic Press, N.Y. 819, 1963.

6. Moore, S. In Chemistry and Biology of Peptides (Johannes Meinhofer Ed.) Ann Arbor Publishers, mich. 629, 1972.

7. Moore, S. On the determination of cystine as cystic acid. J. Biol. Chem. 238: 235, 1963.

8. Ellman, G.L. Tissue sulfhydryl groups Arch. Bio. Chem. Biophys. 82, 70

(1959).

9. Andrews, P. Estimation of molecular weights of proteins by sephadex gel filtration. Biochem. J. 91: 222, 1969.

10. Hirs, C.H.W. Glycopeptides, in Methods of Enzymology 11 (Hirs, C.H.W. Ed.) Academic Press, N.Y. 411, 1967.

11. Warren, L. The Thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234, 1971, 1959.

12. Zawacki, B.E., The Natural History of Reversible Burn Injury. Surgery, Gynecol. and Obstet. 139, 867, 1974.

13. Holleman, W.H. and Weiss, L.J. The thrombin-like enzyme from *Bothrops atrox* snake venom. J. Biol. Chem. 251: 867, 1974.

14. Nolan, D., Hall, L.S., and Barlow, G.H. Ancord, The coagulant enzyme from Malayan Pit Viper (*Agkistodron rhodostoma*) venom. Methods in Enzymology 45: (L. Lorand Ed.) Academic Press, N.Y. 205,1976.

15. Halton, N.W.C. Studies on the coagulant enzyme from *Agkistrodon rhodostoma* venom. Biochem. J. 131: 799, 1973.

16. Markland, F.S. and Pirkle, H. Biological activities and biochemical properties of thrombin-like enzymes from snake venoms. In Chem. and Biol. of Thormbin (R.L. Lundblad, J.W. Fenton and K.G. Mann, Eds) Ann Arbor Science Press, Ann Arbor, Michigan 1977, p. 71.

17. Damus, P.S., Markland, F.S., Davidson, T.M. and Shanley, J.O.: A purified procoagulant enzyme from the venom of the Eastern Diamondback Rattlesnake (Crotalus adamateus) in vivo and in vitro studies. J. Lab. Clin. Med 79: 906 (1972).

18. Wood, S. and Hilgard, P.H.: Arvin-induced hypofibrinogenimia and metastasis formation from blood borne cancer cells. Johns Hopkins Med. J. 133: 207 (1973).

19. Hilgard, P. and Thornes, R.D.: Anticoagulants in the treatment of cancer. Eur. J. Cancer 12: 755 (1976).

20. Ivarsson, L.: Pulmonary metastasis formation after intravenous tumor cell injection in defibrinogenated rats. Zeit Krebsforsch Klinis Onkolgie 85: 83 (1976).

21. Dewys, W.D., Kwaan, H.C. and Bathina, S.: Effect of defibrination on tumor growth and response to chemotherapy. Cancer Res. 36: 3584 (1976).

SIGNIFICANT ACCOMPLISHMENTS

1. A large-scale purification procedure for crotalase was developed and enzyme was recovered in high yield (50%) having high specific activity (407 units/mg).

2. Benzamidine-sepharose affinity chromatography was applied for the first time to purify crotalase.

3. The enzyme was relatively stable to SP-Sephadex column chromatography at pH 5.5 and recovery was better than the previously reported recovery in which this step was performed at lower pH.

4. Analytical isoelectric focusing was conducted for the first time and it showed 5 bands which were shown to be due to differences in sialic acid content of the enzyme.

5. The amino acid composition of crotalase was determined and shown to be similar to that previously established by Markland and Damus (1), the carbohydrate content of crotalase was redetermined and it was shown that enzyme contains 6.1% carbohydrates plus variable amounts of sialic acid up to 6.3%.

6. Fibrinolytic activity was shown to be an inherent property of crotalase since the ratio of fibrinogenolytic activity to fibrinolytic activity remained constant throughout the purification procedure.

7. Amidolytic activity of crotalase was observed on several chromogenic substrates. Since crotalase hydrolysed the thrombin substrate S-2238, we propose to use this substrate for a rapic assay of crotalase during purification or for kinetic studies.

8. We have established that crotalase can be used to defibrinogenate rabbit blood. The optimal dose (50-100 units/kg body weight) can be used in burn repair studies and this dose was successfully applied to maintain defibrinogenation in spite of the increased fibrinogen levels following burns.

9. A suitable mold for causing burn injury in rabbits was preparted and was used to cause third degree burns in these animals.

Publications by the Principal Investigator:

(1) Pirkle, H., Markland, F.S., and Theodor, I.: Thrombin-like enzymes of snake venoms: actions on prothrombin. Thrombosis Research 8: 619-627, 1976.

(2) Markland, F.S.: Purification and properties of crotalase. The thrombin- like enzyme from Crotalus adamanteus. Methods in Enzymology 45: 223-236, 1976.

(3) Markland, F.S. and Pirkle, H.: Thrombin-like enzyme from the venom of the eastern diamondback rattlesnake (Crotalus adamanteus). Thrombosis Research 10: 487-494, 1977.

(4) Markland, F.S. and Pirkle, H.: Biological activities and biochemical properties of thrombin-like enzymes from snake venoms. In Chemistry and Biology of Thrombin (Lundblad, R.L., Mann, K.G., and Fenton, J.W., Eds.), Ann Arbor Science Press, Ann Arbor, Michigan, July, 1977.

(5) Markland, F.S. and Pirkle, H.: Characterization of a thrombin-like enzyme from Crotalus adamanteus (eastern diamondback rattlesnake).

Toxicon (Abstracts) 14:412-413, 1976.

(6) Markland, F.S., Chou, J., Shih, Y. and Pirkle, H.: A new procedure for purification of crotalase from Crotalus adamanteus venom. Thrombosis and Hemostasis (Abstracts) 38: 223, 19771

(7) Bajwa, S.S. and Markland, F.S.: Defibrinogenation studies with crotalase (thrombin-like enzyme from eastern diamondback rattlesnake venom) possible clinical application. Proceedings of Western Pharmacological Society. Tahoe, California. 1978 (Accepted).

(8) Bajwa, S.S. and Markland, F.S.: A new method for the purification of a thrombin-like snake venom enzyme. Thrombosis Research (1978), submitted.

Talks by the Principal Investigator:

(1) "Snake bite victim", Hematology Conference, LAC/USC Medical Center, October 29, 1976, Los Angeles, California.

(2) "Biological activities and biochemical properties of thrombin-like enzymes from snake venoms", Mayo Clinic, Rochester, Minnesota.

(3) "Thrombin-like enzymes from snake venoms", Biochemistry Department Seminar,Colorado State University, June 13, 1977, Ft. Collins, Colorado.

(4) "A new procedure for purification of crotalase from Crotalus adamanteus venom", 6th International Congress on Thrombosis and Hemostasis, July 1, 1977, Philadelphia, Pennsylvania.

(5) "Defibrinogenation studies with crotalase(Thrombin-like enzyme from eastern diamondback rattlesnake venom); Possible clinical applications", Western Pharmacology Society Meeting, January 25, 1978, Tahoe, Nevada.

CONCLUSIONS

Crotalase, the thrombin-like enzyme from eastern diamondback rattlesnake (Crotalus adamanteus), has been purified to homogeneity. This was shown by chromatographic and electrophoretic patterns of the enzyme. A single band was observed on SDS polyacrylamide gel electrophoresis. The molecular weight was estimated to be about 33,000 by gel filtration on Sephadex G-100 and SDS polyacrylamide electrophoresis. The molecular weight determined from amino acid composition was shown to be 30,334. However, the estimated molecular weight contributed by carbohydrates (2013) gave us an overall value of 32,347. Analytiical isoelectric focusing of crotalase showed five bands. This heterogeneity of enzyme may be due to differences in sialic acid content of the different forms of the enzyme. Upon treatment of the enzyme with neuraminidase to remove the sialic acid a single band was obtained by isoelectric focusing, supporting our contention that the enzyme is homogeneous and that the five bands represent different levels of sialylation of the enzyme. This heterogeneity may have been an artifact of preparation.

Fibrinolytic activity of crotalase was shown to be an inherent property since the ratio of fibrinogenolytic activity tyo that of fibrinolytic activity remained constant troughout the purification procedure. Crotalase hydrolysed a chromogenic substrate (S-2238) used for thrombin assay; this activity could be used as a method for the estimation of crotalase.

We have utilized crotalase to investigate the possible application of this enzyme in clinical situations using experimental animal systems. We have developed a model system utilizing the rabbit to investigate the effect of defibrinogenation on burn injury and repair. Dose response studies have been performed to determine

the optimum level of crotalase to defibrinogenate rabbits during burn trauma.

Control studies have shown that plasma levels of fibrinogen rise from 100% in normal to 250% following 5% total body burn. In non-traumatized rabbits intravenous infusion of 4-6 NIH units of crotalase per kilogram body weight per hour produced a defibringenated state which lasts for 6-12 hours. In burned animals, daily infusion of crotalase (50-100 units/kg body weight) has successfully been employed to maintain defibrinogenation in spite of the increased fibrinogen levels following burns. In further experiments we propose to evaluate the effects of defibrinogenation on burn repair using revascularization in burned area as an index of wound healing.

Figure Legends

Fig. 1 Elution Profile of crude Crotalus adamanteus venom from Sephadex G-100 column: Elution was performed with 0.04 M Tris-HCl (pH 7.1) containing NaCl (0.1M). Column Size was 84 x 2.5 cm and flow rate was 25 ml/h. Fractions of 11.0 ml were collected, the absorbance at 280 nm determined and fibrinogen clotting activity measured.

Fig. 2 DEAE-Cellulose column chromatography of fraction containing crotalase after Sephadex G-100 column: The dialyzed (against 0.005 M sodium acetate buffer, pH 7.0) G-100 fraction was loaded on a DEAE-Cellulose column (2.5 x 40 cm) and eluted with the dialyzing buffer (100 ml), followed by the first linear gradient established with 425 ml of the same buffer and 425 ml of 0.1 M sodium acetate buffer (pH 7.0). The second gradient was made by 400ml of 0.7M sodium acetate buffer (pH 7.0). Fractions of 9.0 ml were collected at a flow rate of 20 ml/hr.

Fig. 3 Elution profile of benzamidine affinity column: The enzyme after DEAE-Cellulose chromatography was dialyzed against 0.05 M Tris-HCl-0.15M NaCl (pH 9.0) and loaded onto banzamidine affinity column (1.5 x 30 cm) and eluted with 80 ml of the dialysis buffer, then 200 ml of 0.05M Tris-HCl-0.4M NaCl (pH 9.0) and finally 250 ml of 0.05M Tris-HCl-0.1M NaCl-0.15M banzamidine (pH 9.0). Fractions of 3.0 mlwere collected at a flow rate of 10 ml/hr.

Fig. 4 Elution profile of SP-Sephadex C-50column chromatography: The fraction containing crotalase activity after affinity chromatography was dialyzed against sodium acetate buffer, 0.05M (pH 5.5) and loaded on SP-Sephadex (1.5 x 30 cm) and eluted with 140 ml of the dialysis buffer. The initial gradient employed 75 ml of starting buffer and 75 ml of 0.1M sodium acetate buffer (pH 5.5) and the second gradient

employed 100ml of 0.1M acetate buffer (pH 5.5) and 100 ml of 0.7M acetate buffer (pH 5.5). Fractions of 2.1 ml each were collected at a flow rate of 9.0 ml/hr.

Fig. 5 SDS gel electrophoresis of crotalase preparations after different chromatographic steps: The method has been described in text A. crude venom B. after G-100 C. after the DEAE-cellulose D. after the Benzamidine Sepharose E. After the SP-Sephadex.

Fig. 6 Molecular Weight determination of purified crotalase by Sephadex G-100: Sephadex G-100 column (90 x 1.75 cm) was equilibrated with 0.05M Tris-HCl-0.15M NaCl (pH 9.0) and standardized with standard proteins: Aldolase, 158,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; and ribonuclease A, 13,500. Void volume was estimated by using the equilibrating buffer. Fractions of 3 ml each were collected at a flow rate of 25 ml/hr. Crotalase was separately loaded and eluted and assayed for clotting activity. The molecular weight was calculated from the plot of Ve/Vo versus the known molecular weight of the standard proteins.

Fig. 7 Molecular weight estimation of purified crotalase by SDS electrophoresis: SDS electrophoresis was carried on as described in methods and the marker proteins were used as shown in Fig. 6. The molecular weight of crltalase was calculated from the plot of mobility versus known molecular weight of standard proteins.

Fig. 8 Effects of neuraminidase on the amidolytic activity of crotalase: The complete reaction mixture consisted of 1 ml of (0.05M Tris-0.125M NaCl-0.025M HCl) buffer, 1.25 units of crotalase, 0.1 ml of S-2238 (1 mmol./1), with or without 1.25 units of neuraminidase in a total volume of 1.25 ml. The reaction mixture was incubated at 37°C and the liberated p-nitroaniline was read at 405 nm at different time intervals

over a 10 minute period.

Fig. 9 Hydrolysis of chromogenic substrates with different concentrations of crotalase: The preincubation mixture consisted of 2.2 ml of (0.05M Tris-0.125M NaCl-0.025M HCl, pH 8.3) buffer and 0.1 ml of enzyme. The mixture was preincubated at 37°C and 0.2 ml (1 mmol/l) substrate was added and incubated for 3 minutes. The reaction was stopped by adding 0.1 ml of 50% ACOH. p-Nitroaniline color was followed at 405 nm.

Fig. 10 Hydrolysis of S-2238 with different concentrations of crotalase: The complete reaction mixture consisting of 1.0 ml of buffer (0.05M Tris-0.125M NaCl-0.025M HCl, pH 8.3) and 0.15ml of different concentrations of enzyme and mixed and preincubated at 37°C. S-2238 (0.01 ul,1 mmol/l) was added and incubated for 3 minutes and the liberated p-nitroaniline was followed at 405 nm. The reaction was stopped by adding 0.05 ml of acetic acid.

Fig. 11. Defibrinogenation of rabbits by injecting crotalase within 5 minutes: Crotalase at different concentrations was injected into rabbits as described in the text. Blood samples were taken before enzyme infusion and at 2 hour intervals and fibrinogen estimated as described in the methods.

Fig. 12. Rabbit defibrinogenation by 1 hour I.V. infusion of crotalase: Crotalase (6 units/kg body weight) was infused over a one hour period into rabbits as described in the text. Blood samples were taken prior to crotalase infusion and at 2 hourintervals following infusion. Fibrinogen levels were determined and are reported in % relative to the zero time control.

Fig. 13 Rabbit defibrinogenation by 1 hour I.V. infusion of crotalase: Crotalase (100 units/kg body weight) was infused and blood samples were taken at the times indicated. Fibrinogen was determined and plotted as described in the legend to Fig. 11.

Fig. 14 Rabbit defibrinogenation by daily 1 hour I.V. infusion of crotalase:

A blood sample was taken at zero time and crotalase (100 units/kg body weight) was then infused. Daily 1 hour infusions were repeated as indicated by the arrows. Fibrinogen was estimated daily and plotted as described in the legend to Fig. 11.

Fig. 15 Burned rabbit defibrinogenation by daily 1 hour I.V. infusion of crotalase:

Two rabbits (8.5 lbs) were burned as described in the text. Blood samples for fibrinogen estimation were taken as shown in the figure and crotalase (100 units/kg body weight) was injected immediately into one of the rabbits and daily infusions were continued throughout the course of the experiment as indicated by the arrows.

TABLE 1

PURIFICATION OF CROTALASE

PURIFICATION STEP	TOTAL PROTEIN (MG)	Activity (UNITS)	SP. ACT. (UNITS/MG)	% RECOVERY
(1) Crude Venom	3,587.8	28,000	7.4	100.0
(2) Gel Filtration	1,625.0	26,250	16.1	93.7
(3) DEAE-Cellulose Chromatography	1,118.8	25,840	23.1	92.2
(4) Benzamidine Sepharose Affinity Chromatography	175.8	22,500	127.9	80.3
(5) SP-Sephadex Chromatography	34.9	14,196	407.0	50.7

TABLE II

Comparison of Fibrinogenolytic and Fibrinolytic Activity of Crotalase

Specific Activity	Fibrinogenolytic Activity	Fibrinolytic Activity	Ratio
	Units/mg protein	Units/mg protein	
Snake Venom	16.23	0.18	90
Sephadex G-100	32.37	0.34	95
DEAE-Cellulose	38.50	0.40	96
Benzamidine Sepharose	189.60	2.07	92
SP-Sephadex	433.60	4.92	88

TABLE III

AMINO ACID COMPOSITION OF CROTALASE

Amino Acid	24-hour Average	Ratio to Alanine	48-hour Average	Ratio to Alanine	72-hour Average	Ratio to Alanine	Average or Extrapolated Ratio	Minimum Residue Weight	Residues (Average Ratio times factor)	Integral number of Residues	Molecular Weight
ASP	136.5	1.97	134.1	2.10	252.02	1.95	2.01	231.2	27.69	28	3,220
THR	66.5	0.96	65.2	1.02	129.7	1.00	1.02[b]	104.0	14.05	14	1,428
SER	99.6	1.44	85.2	1.33	168.2	1.30	1.48[b]	130.3	20.39	20	1,760
GLU	131.0	1.89	121.5	1.90	249.6	1.93	1.91	243.3	26.31	26	3,380
PRO	121.3	1.75	111.0	1.74	205.5	1.59	1.69	165.6	23.28	23	2,254
GLY	114.6	1.66	105.9	1.66	220.6	1.70	1.67	106.9	23.00	23	1,472
ALA	69.2	1.00	63.9	1.00	129.5	1.00	1.00	72.0	13.78	14	1,008
VAL	64.5	0.93	68.8	1.08	139.4	1.08	1.08[c]	103.0	14.88	15	1,500
ISL	67.7	0.97	76.1	1.19	146.5	1.13	1.13[c]	125.4	15.57	16	1,824
LEU	95.4	1.38	99.3	1.55	180.5	1.39	1.44	164.2	19.84	20	2,280
TYR	21.1	0.30	17.6	0.28	23.8	0.18	0.30[e]	49.2	4.13	4	656
PHE	67.5	0.97	66.3	1.04	116.9	0.90	0.97	143.6	13.36	13	1,924
HIS	36.9	0.53	38.7	0.61	78.95	0.61	0.58	81.2	7.99	8	1,120
LYS	48.3	0.69	49.0	0.77	103.0	0.80	0.75	97.5	10.33	10	1,300
ARG	53.2	0.77	51.8	0.81	106.6	0.82	0.80	126.4	11.02	11	1,738
HALF-CYS							1.48[d]	223.4	20.31	20	3,020
MET							0.29[d]	38.3	3.99	4	528
TRP							0.35[a]	65.1	4.82	5	930
	Sum							2,248.1		274	30,334

[a] *Determined after hydrolysis with* 4N *methane sulfonic acid reagent containing* 0.2% 3-(2-aminoethyl)-indole for 20 hours at 115°C in duplicate.

[b] Determined by extrapolation to zero time.

[c] Determined from values obtained after 72 hour hydrolysis.

[d] Halfcystine determined as cysteic acid and methionine as the sulfone after performic acid oxidation (15).

[e] Determined from values obtained after 24 hour hydrolysis.

TABLE IV

Effect of Inhibitors on the Amidolytic and Clotting Activity of Crotalase

Name	Amount Used		% Inhibition	
			Amidolytic Activity	Clotting Activity
Pancreatic Trypsin Inhibitor	100	mg	0	0
α_1-Trypsin Inhibitor	1	mg	0	0
Soybean Trypsin Inhibitor	1	mg	0	0
Ovomucoid	5	mg	0	0
Lima bean Trypsin Inhibitor	1	mg	0	0
Hirudin	5	units	0	0
p-amino benzamidine	5	mg	25.6	26.7
m-amino benzamidine	5	mg	87.9	100.0
Benzamidine	5	mg	83.7	2.3
L. Arginine-L-aspastate	4	mg	0	0
Agmatine	4	mg	0	0

FIG. 1

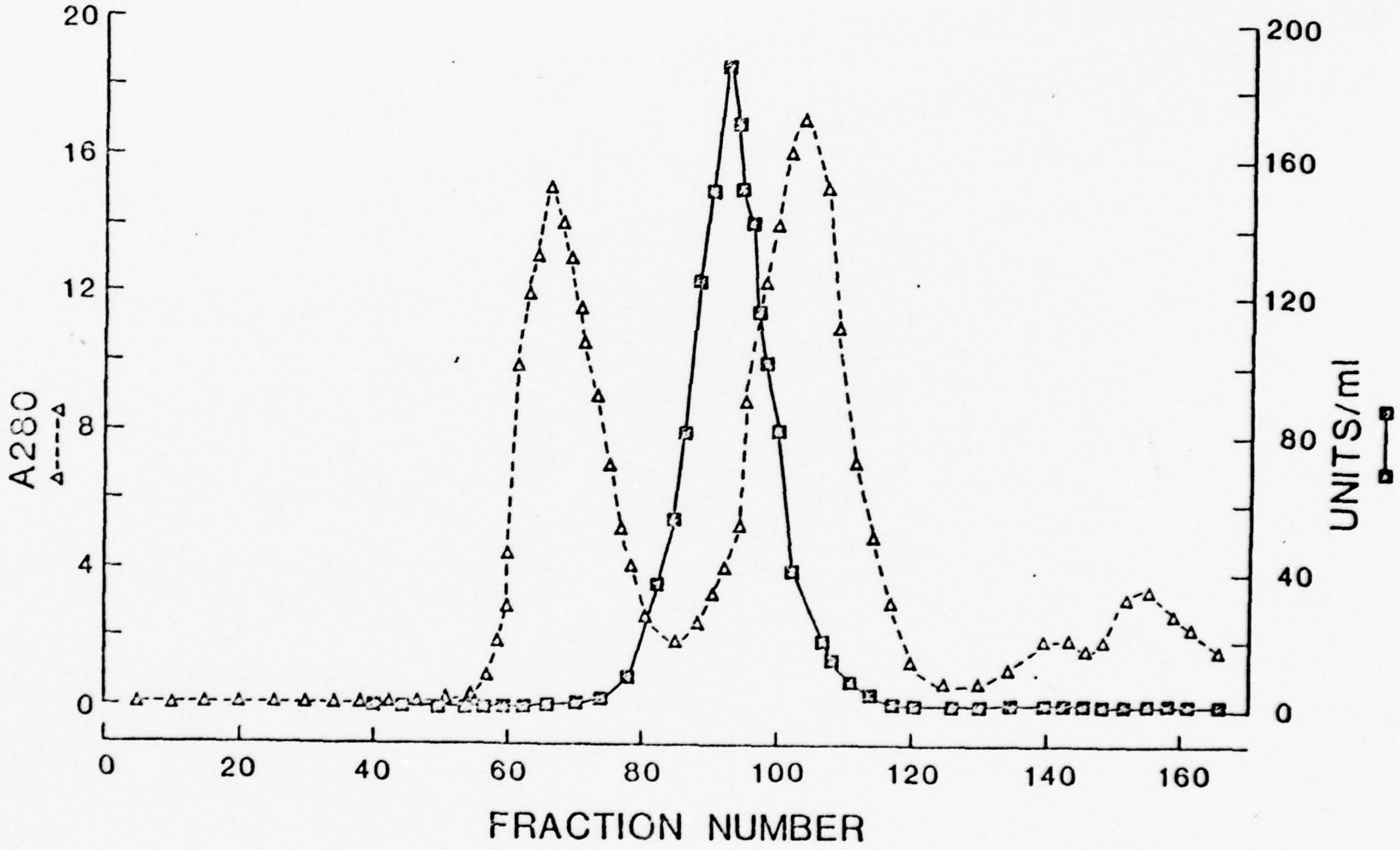


FIG. 2

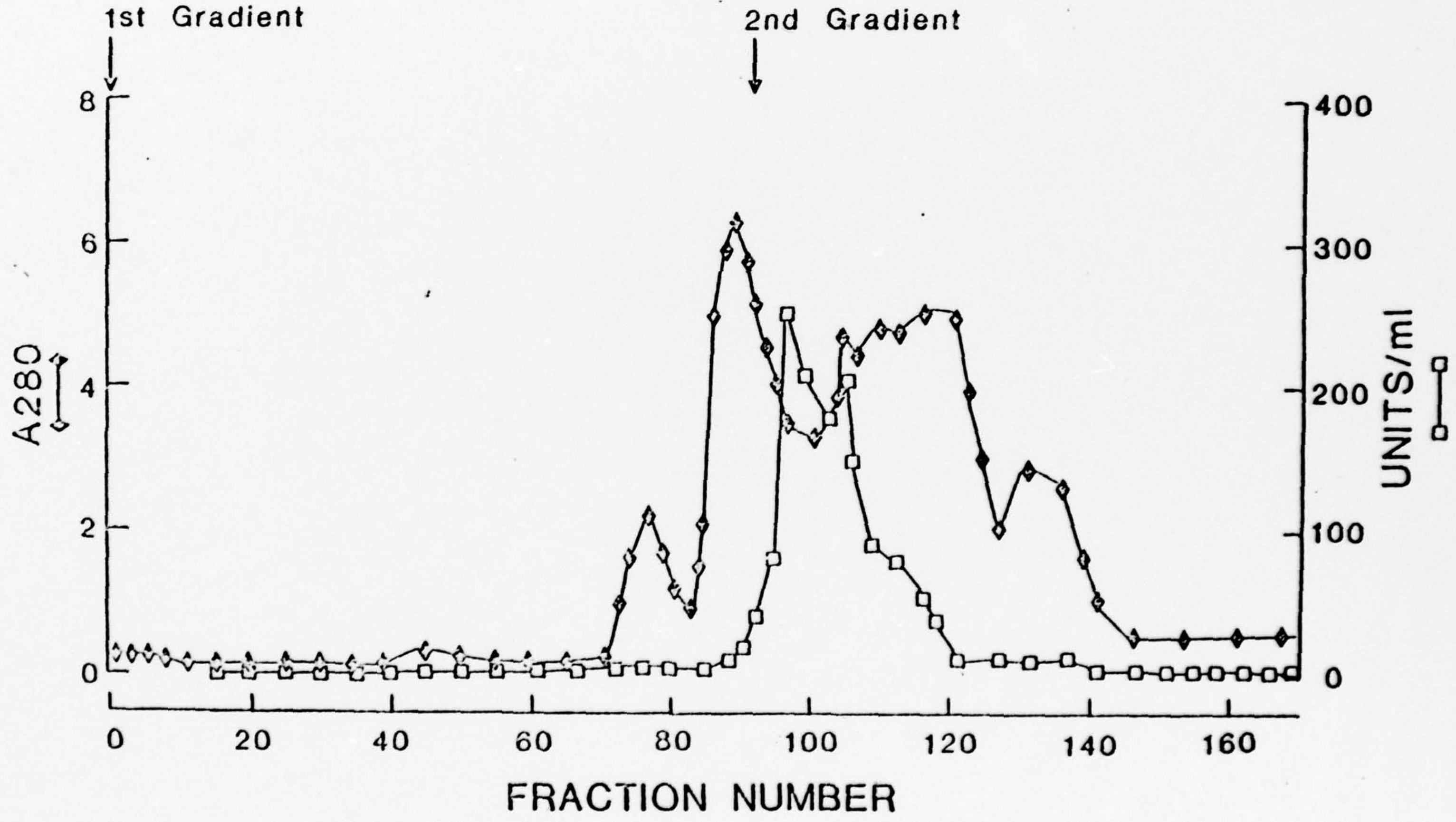


FIG. 3

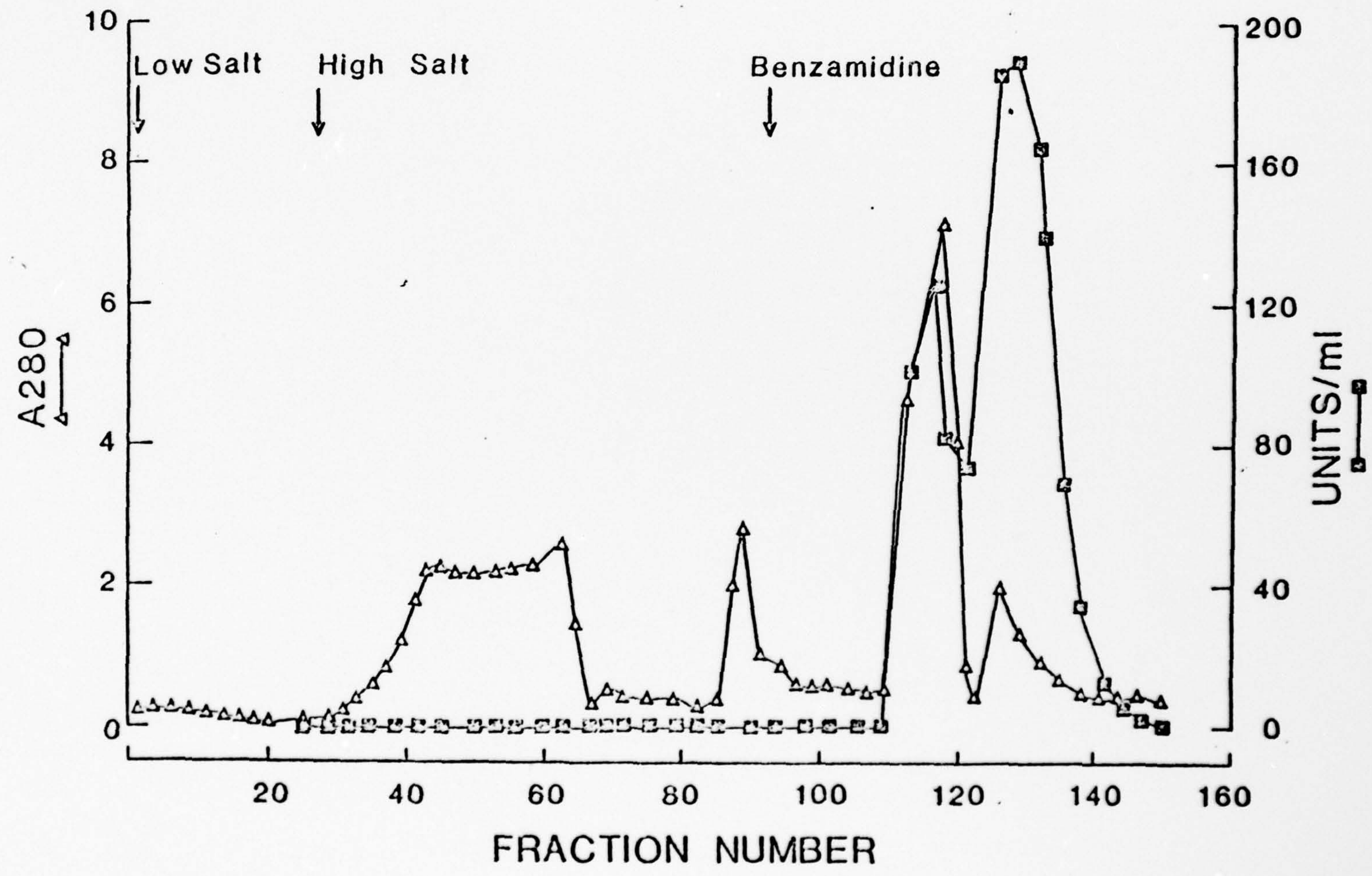


FIG. 4

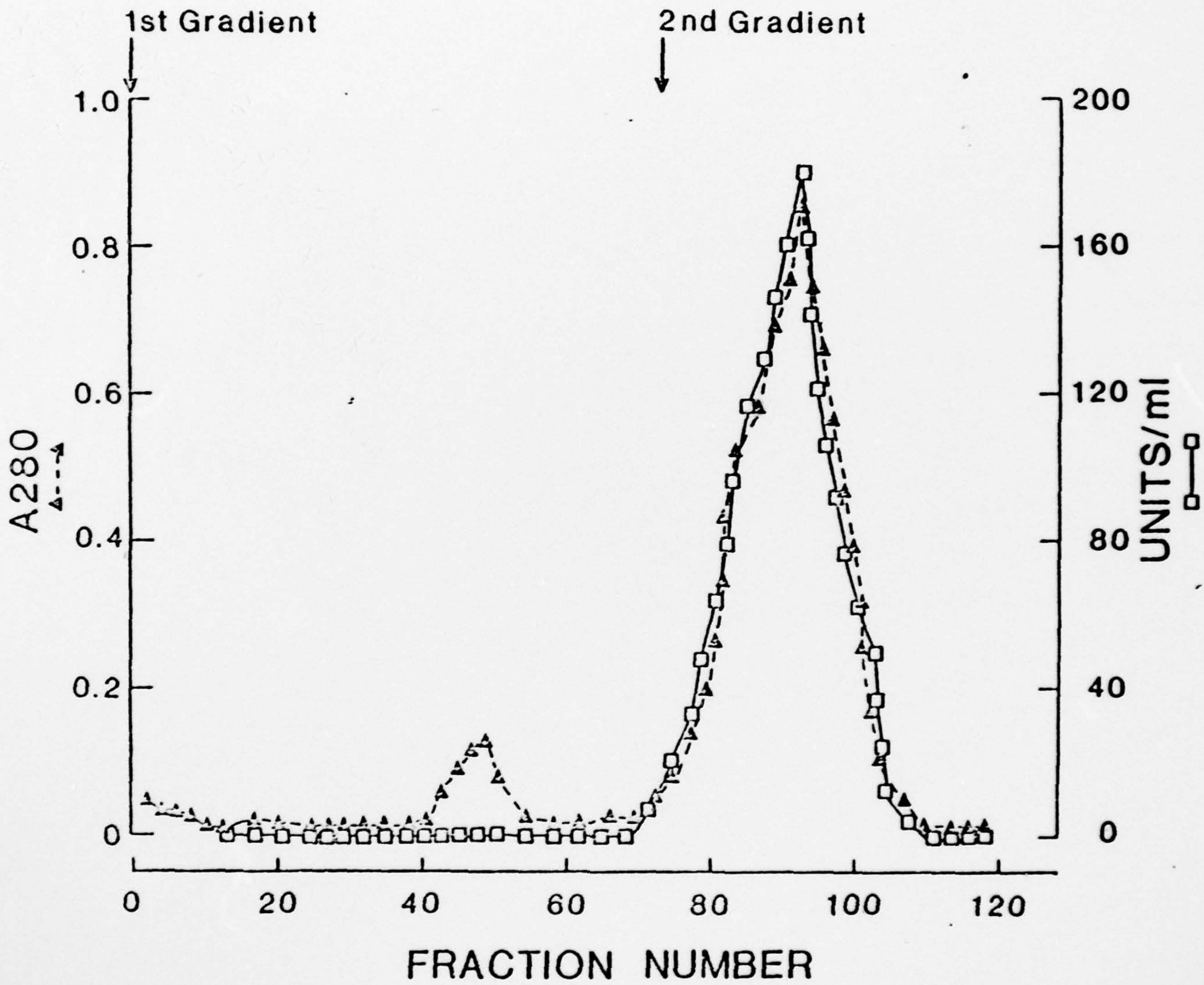


FIG. 5

SDS ELECTROPHORESIS OF CROTALASE PREPARATIONS AFTER

DIFFERENT COLUMN CHROMATOGRAPHY STEPS

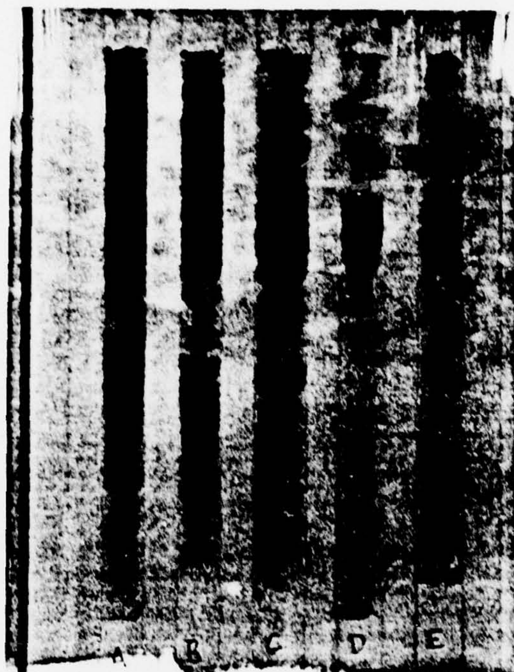


FIG. 6

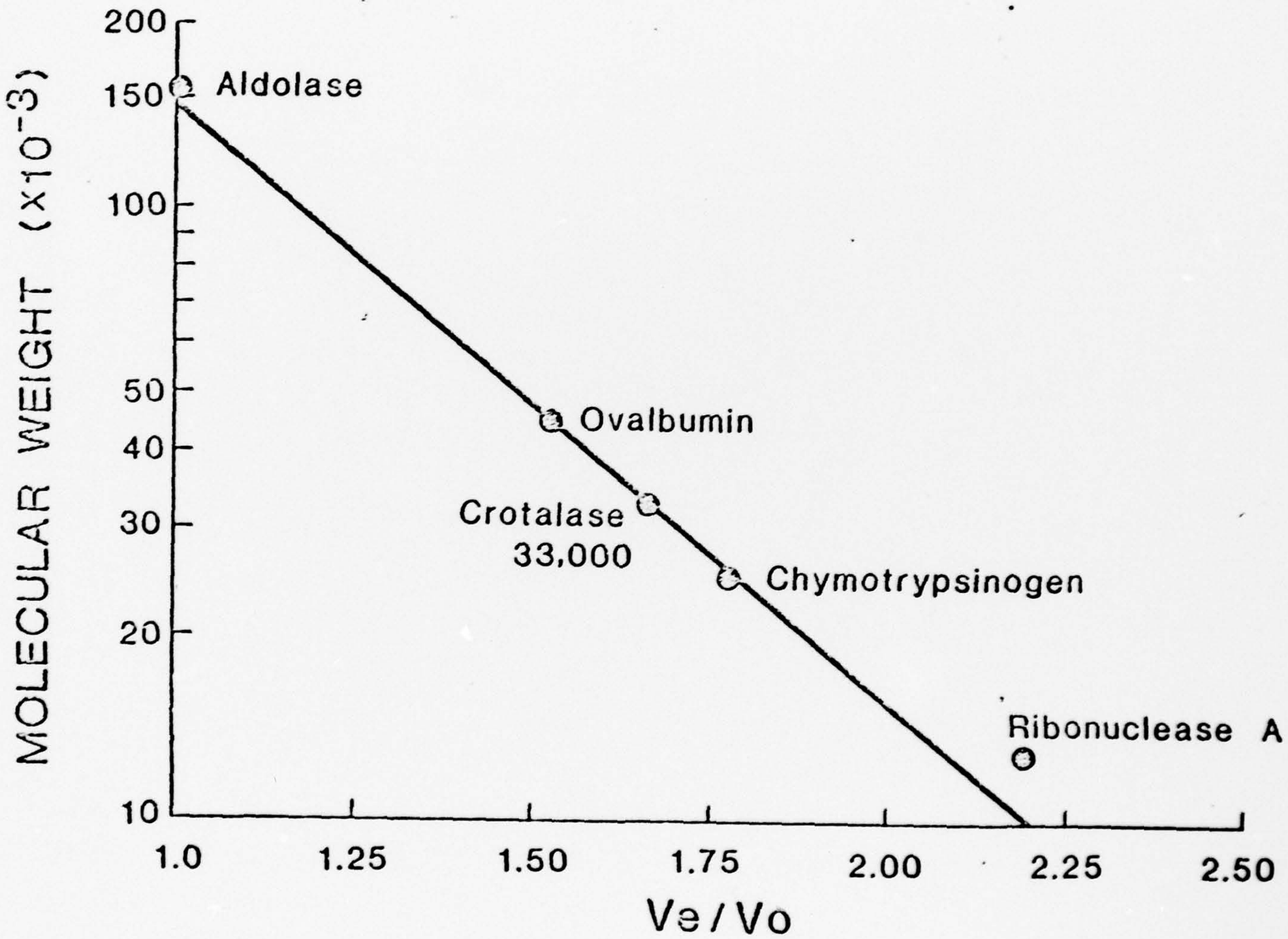


FIG. 7

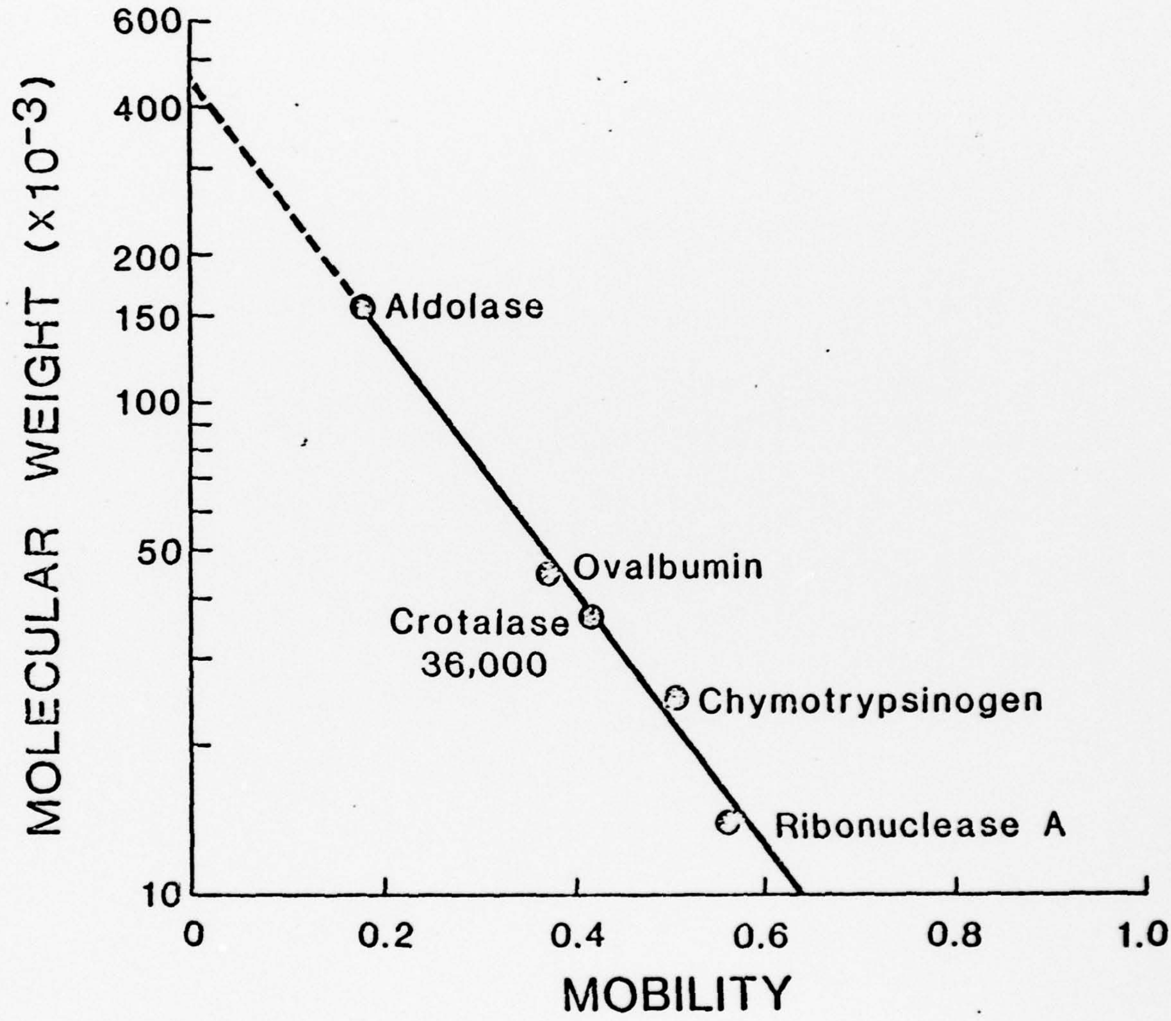


Figure 8

Effect of Neuraminidase on the Activity of Crotalase Towards S-2238

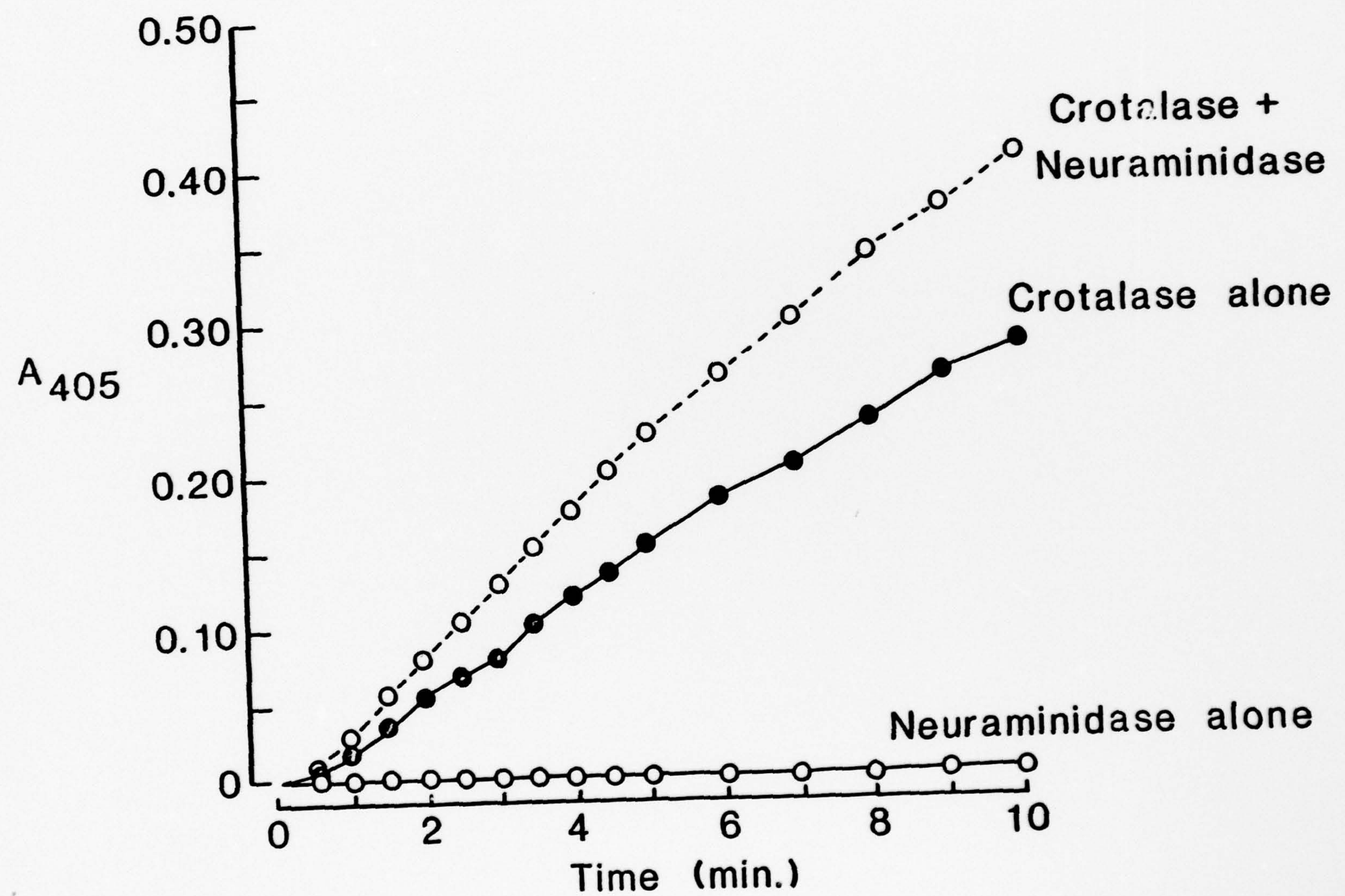


Figure 9
Hydrolysis of Chromogenic Substrates
With Crotalase

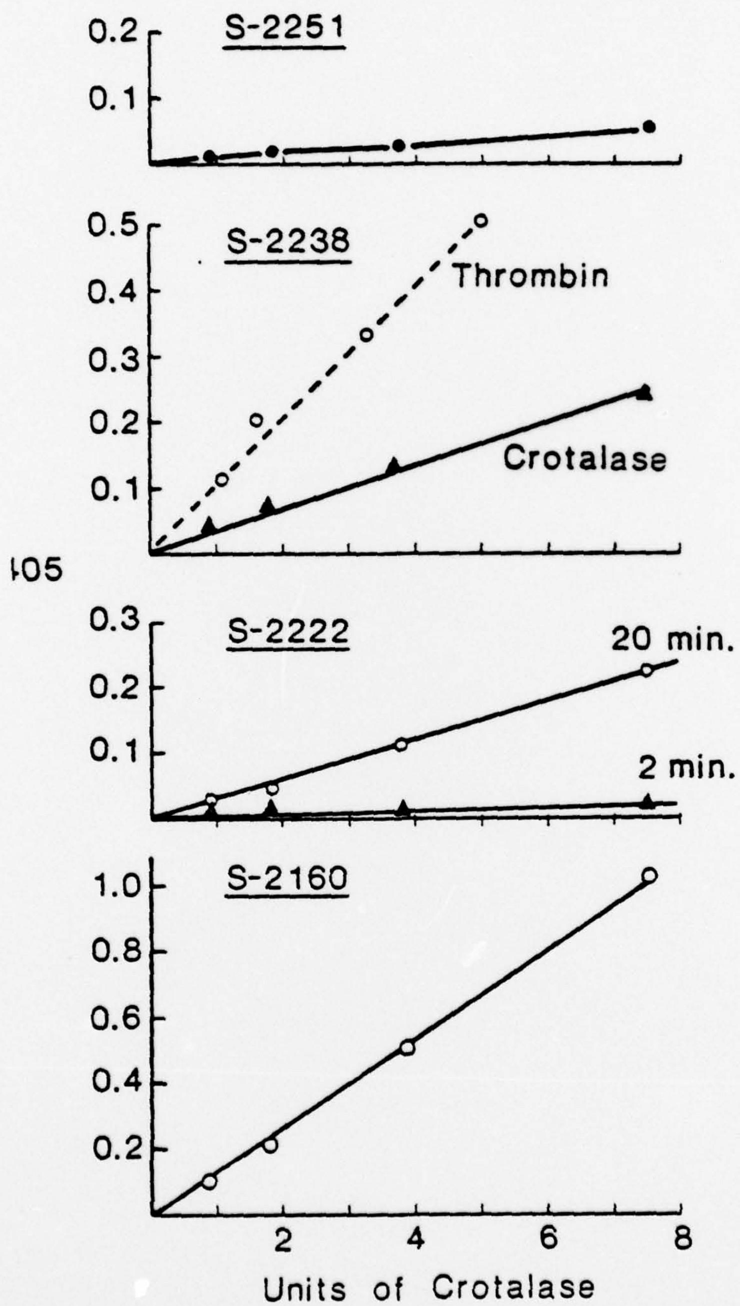
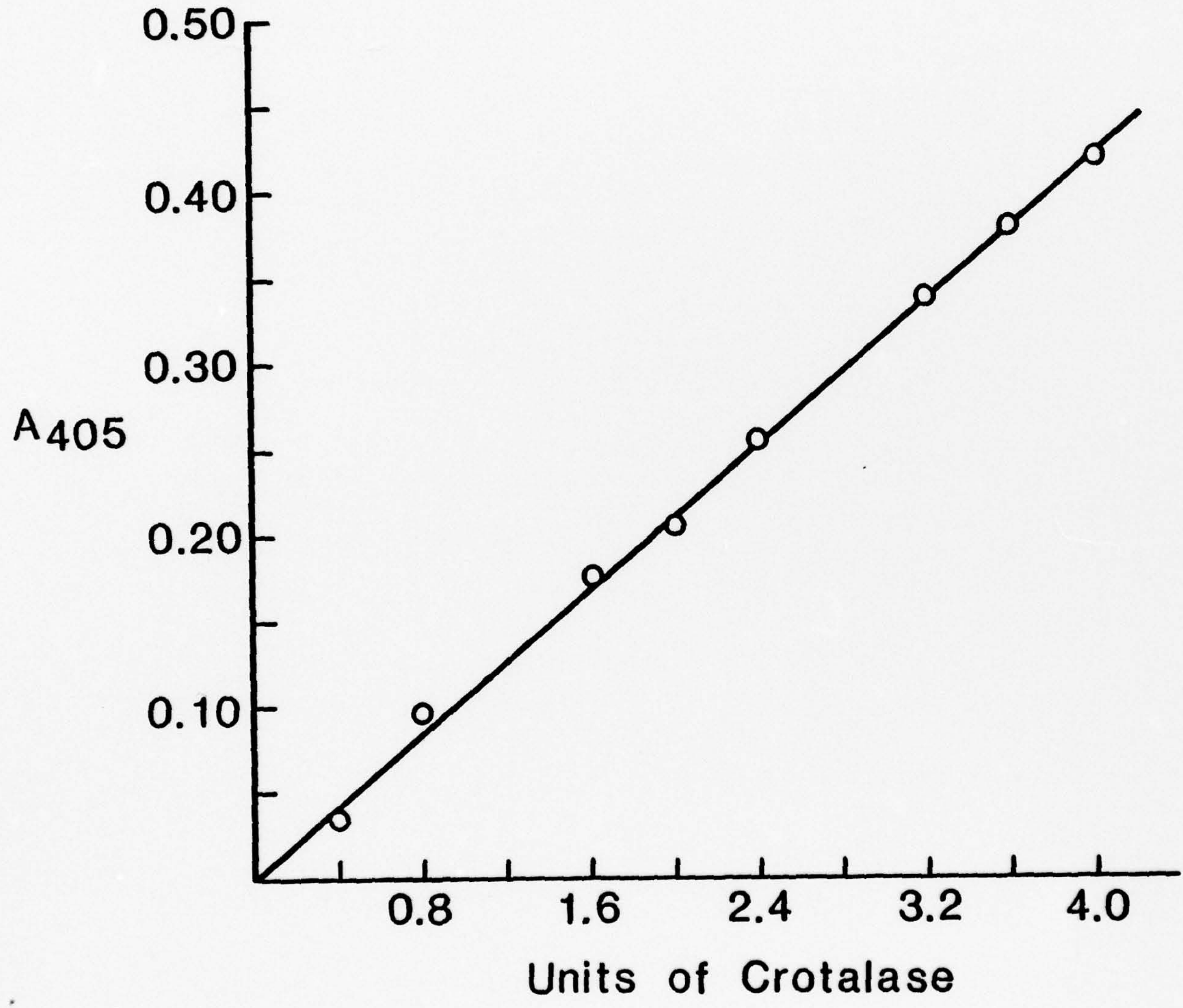


Figure 10
Hydrolysis of S-2238 by
Different Concentrations of Crotalase



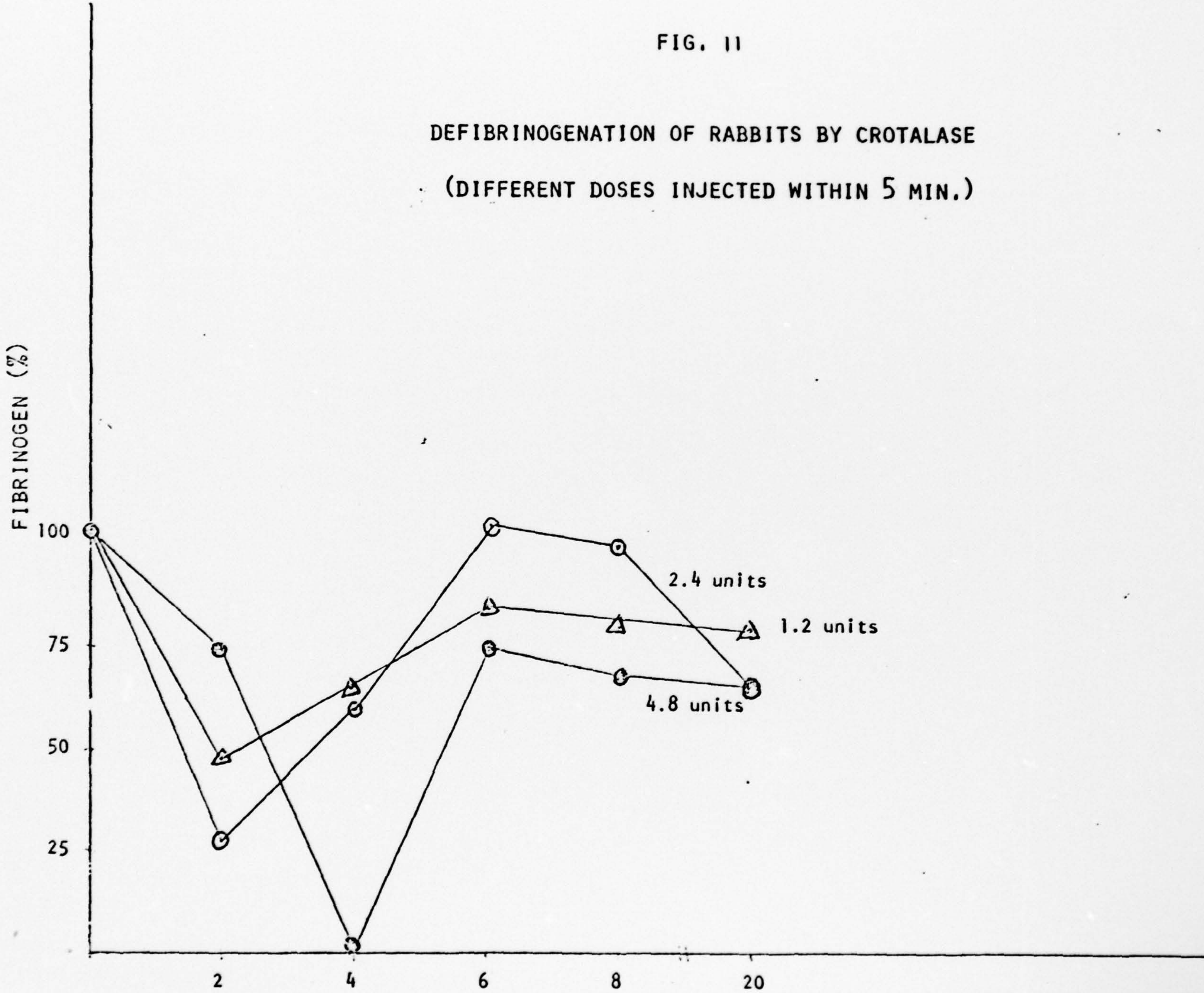


FIG. 11

DEFIBRINOGENATION OF RABBITS BY CROTALASE

(DIFFERENT DOSES INJECTED WITHIN 5 MIN.)

FIG. 12

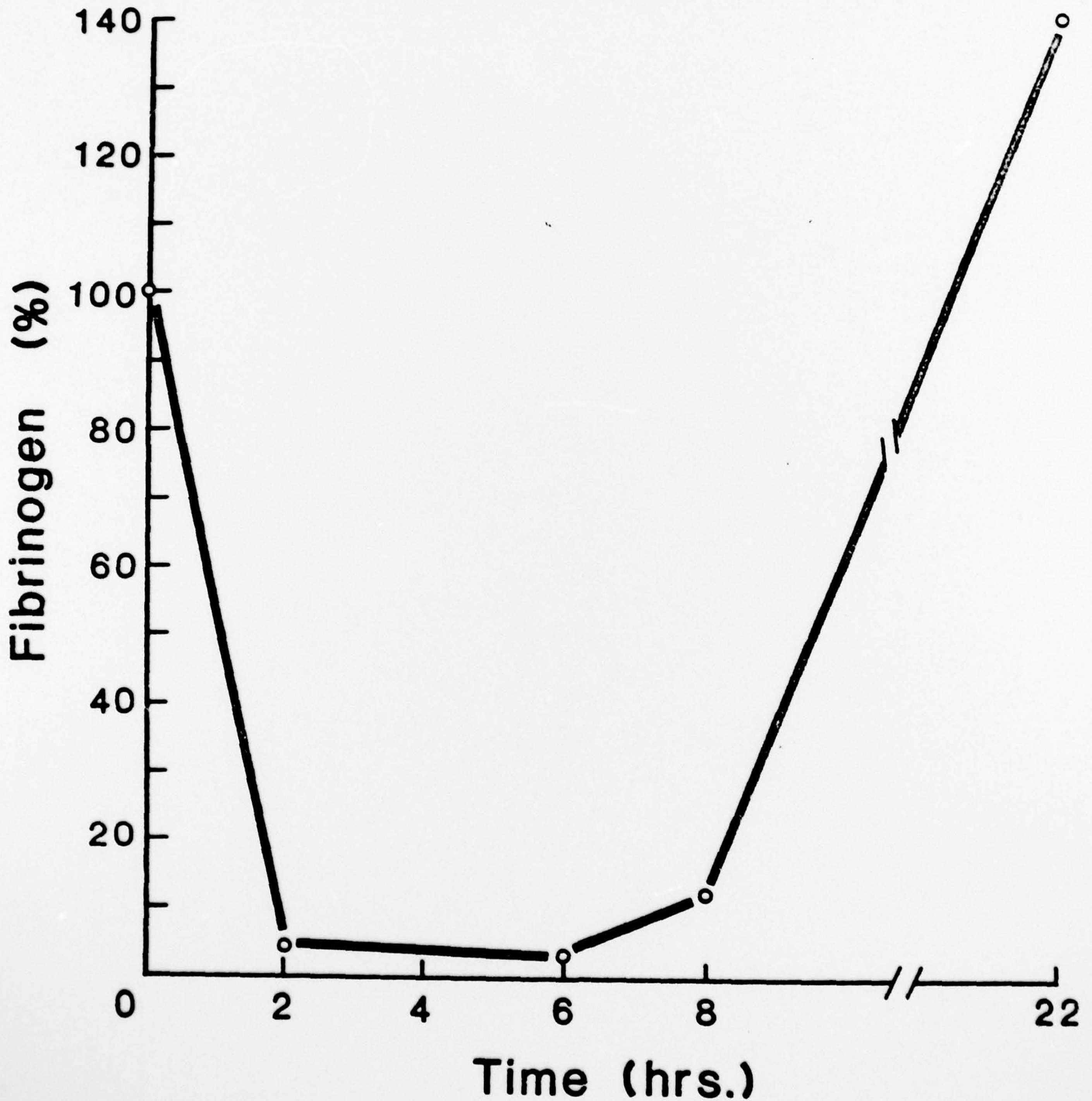


FIG. 13

RABBIT DEFIBRINOGENATION

BY 1 HR. IV INFUSION OF CROTALESE

(100 units/kg)

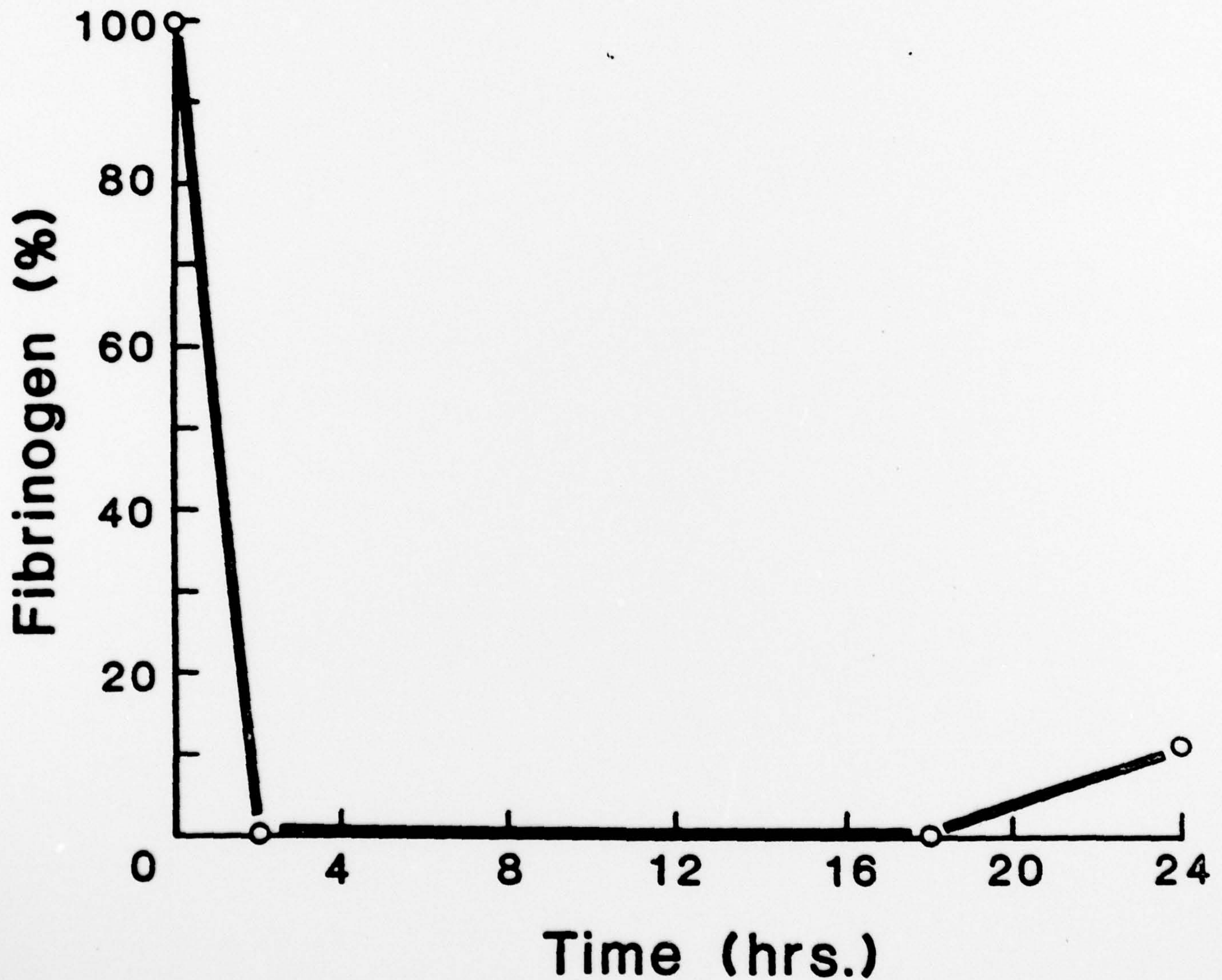


FIG. 14

RABBIT DEFIBRINOGENATION

BY DAILY 1 HR. INFUSION OF CROTALASE

(100 units/kg)

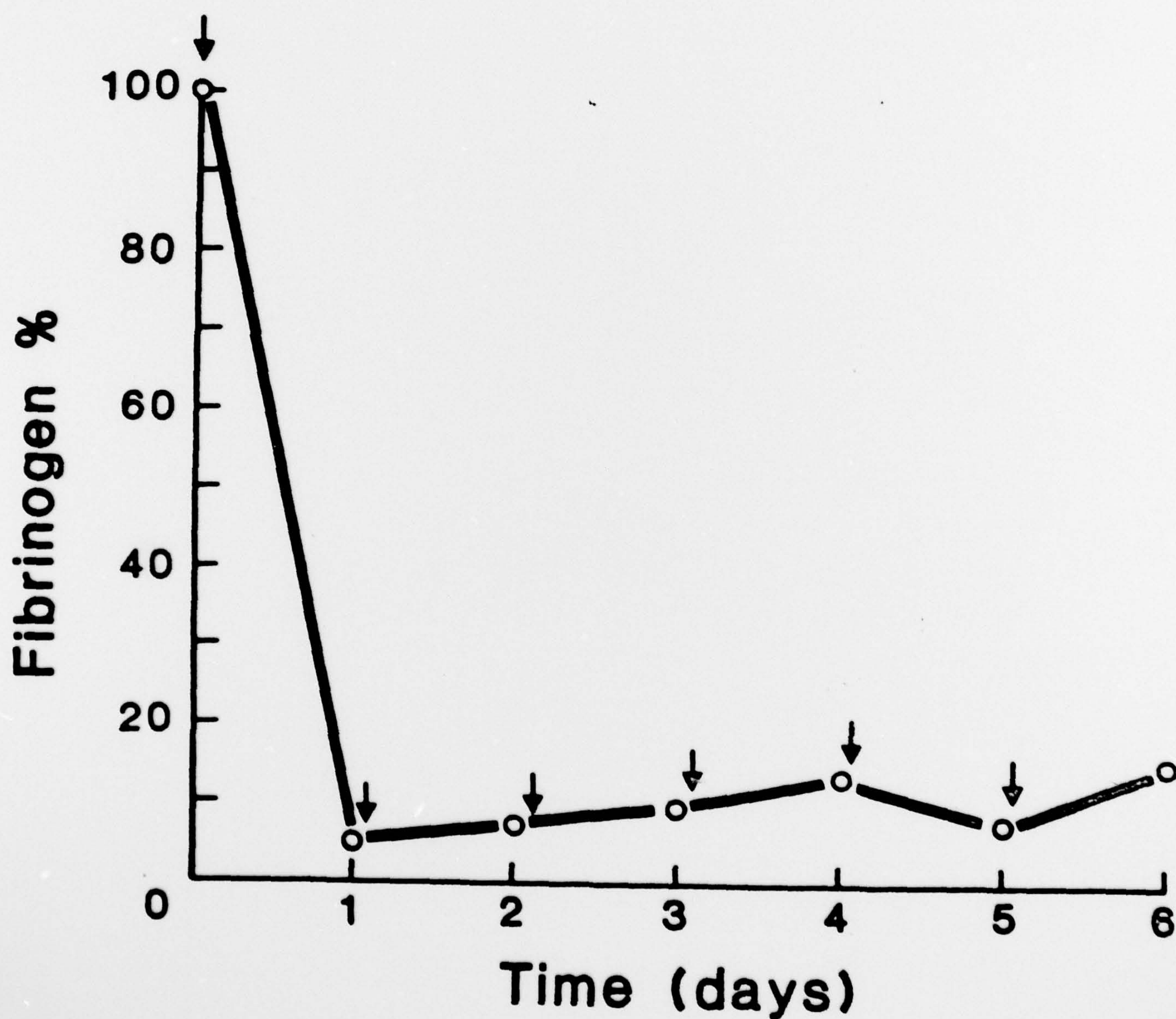


FIG. 15

BURNED RABBIT DEFIBRINOGENATION

BY DAILY 1 HOUR IV INFUSION OF CROTALASE

(100 units/kg)