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MICE PLASMA FIBRINOGEN CONSUMPTION BY THROMBIN-LIKE ENZYME PRESENT IN RATTLESNAKE VENOM FROM THE NORTH-EAST REGION OF ARGENTINA

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Abstract Due to variability of venom components from the same species of snakes that inhabit different regions, particular properties of the venom of *Crotalus durissus terrificus* that inhabits the North-East of Argentina were studied. Gyroxin, a thrombin-like enzyme, was isolated from this venom by gel filtration and affinity chromatography, it was found to be homogeneous according to SDS-PAGE, with a molecular weight of 33 kDa. "Gyroxin syndrome" in mice was tested and it showed changes in the animal behavior, confirming that the isolated thrombin-like enzyme is gyroxin. Effects of this enzyme and the crude venom on mice plasmatic fibrinogen levels were determined. The mice plasma fibrinogen decreased rapidly until incoagulability during the first hour after thrombin-like enzyme injection, then reaching its normal level 10 hours after injection; whereas crude venom resulted in a 60% decrease of the mice plasma fibrinogen, reaching its normal level after the same period of time. After 1 hour of gyroxin inoculation, intravascular coagulation was observed in histological cuttings of lung, cardiac muscle and liver. The isolated enzyme showed strong hydrolyzing activity on fibrinogen and fibrin *in vitro,* whereas the crude venom exhibited weak hydrolyzing activity on both substrates. It is probable that this very low activity is due to the low percentage of the enzyme in the crude venom. Decreasing of plasmatic fibrinogen levels may be due to either the coagulant or hydrolyzing actions of the enzyme.

Key words: *Crotalus durissus terrificus,* venom; North-East Argentina, thrombin-like, coagulation

Resumen ***Consumo de fibrinógeno plasmático en ratones por acción de enzima con actividad trombínica presente en el veneno de cascabel del nordeste argentino***. Teniendo en cuenta la variabilidad de los componentes del veneno de serpientes de una misma especie que habitan regiones diferentes, se decidió estudiar las propiedades particulares del veneno de *Crotalus durissus terrificus* que habita el nordeste de Argentina, Giroxina, una enzima con actividad trombínica, fue aislada del veneno por cromatografía de filtración por gel y de afinidad; se comprobó su homogeneidad y se determinó su peso molecular, 33 kDa, por SDS-PAGE. Se ensayó el síndrome giroxina en ratones, los que mostraron cambios en el comportamiento, confirmando que la enzima tipo trombina aislada es giroxina. Se evaluó la acción de esta enzima sobre los niveles de fibrinógeno plasmático en ratones, comparándola con la del veneno crudo. Se comprobó que la enzima provoca una disminución de los niveles plasmáticos de fibrinógeno hasta la incoagulabilidad, durante la primer hora de inoculación, mientras que el veneno entero produjo una reducción del nivel plasmático en un 60%; sin embargo, en ambos casos, se evidenció una rápida reposición de fibrinógeno plasmático, alcanzando sus valores normales en un plazo de 10 horas. Se observó coagulación intravascular con la administración de giroxina una hora después de la inoculación, evidenciados en estudios histológicos de tejido pulmonar, cardíaco y hepático. En ensayos realizados *in vitro*, la enzima aislada mostró capacidad de degradar fibrinógeno como así también coágulos de fibrina, mientras que el veneno exhibió débil actividad hidrolítica sobre ambos sustratos. Es probable que esta baja actividad sea debida a la baja concentración de la enzima en el veneno. La disminución de los niveles de fibrinógeno plasmático observado en ratones se debería a la acción coagulante de la enzima, sin embargo no se descarta que también contribuya a este proceso una acción hidrolítica sobre fibrinógeno y fibrina por parte de la enzima.

Palabras clave: *Crotalus durissus terrificus,* veneno, Nordeste de Argentina, enzima tipo trombina, coagulación

Blood coagulation by snake venom action was described at the beginning of last century[1]. Thrombin-like enzymes from venoms of snakes belonging to the Viperidae family have been isolated and characterized[2-6].These enzymes are of great interest due to their use as anticoagulants in thrombotic diseases[7].

Altered blood coagulation is not a major factor among the signs and symptoms of the envenoming by South American rattlesnake *(Crotalus durissus terrificus).* The clotting activity of this crude venom has been investigated

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in vitro and was shown to be thrombin-like[8]. Then, the enzyme responsible for this activity was isolated and characterized by Raw *et al* [5]. This enzyme showed to be Aα fibrinogenase on human fibrinogen.

Alexander *et al.*[9] demonstrated that a thrombin-like enzyme from *C. d. terrificus* venom produces the gyroxin syndrome. This thrombin-like enzyme presents the barrel rotation syndrome in mice and has a variety of symptoms; some authors described it as a rolling behavior alternated with prostration periods[10]**.** Other authors mention that the animals show hypoactivity followed by loss of righting reflex, opisthotonos, spastic distortions and rotation around the long axis[9]. However, in some animals the rolling behavior was not observed but symptoms such as a brief period of hyper excitation and running, or paleness and tachypnea. Some mice show immobility, stretching of posterior limbs and grooming behavior. On the other hand, some of the animals die and others remain asymptomatic[11].

Differences in toxic properties are detected in venoms of the same species that inhabit different geographic regions, different species in the same regions, and among newborn and adult specimens[12, 13, 14]. In the North-East region of Argentina the prevalence of accidents caused by Crotalus is low, but its lethality is very high due to the action on nervous system[15], for that reason it is important to know in detail the envenoming physiology caused by species of this region in order to apply an efficient treatment.

In the coagulation, it is essential to know the periods in which this venom can affect blood clotting and the time that the liver spends to re-synthesis fibrinogen. It is also important to analyze if the low affectation of the clotting system is due to the low activity of the major responsible enzyme (thrombin-like enzyme) or to the low concentration of this enzyme in the venom.

In this paper we describe the *in vivo* effect of *C.d. terrificus* venom from the North-East region of Argentina and the purified thrombin-like fraction on plasmatic fibrinogen levels, determining if the low injury on system is due to the low activity of the thrombin-like enzyme or to its low concentration in the venom. At the same time, the aim of this work is to distinguish if the consumption of fibrinogen is due only to the coagulant action of the enzyme or to both the coagulant and fibrinogen-fibrin hydrolyzing activities of the enzyme.

Materials and Methods

Chemicals and reagents

Crotalus durissus terrificus adult snakes proceeding from the North-East region of Argentina were kept in the Serpentarium of the local zoo, Corrientes, Argentina. Venom was collected, desiccated and immediately stored at –20 °C. Molecular weight markers (pre-stained SDS-PAGE standards, low range) were purchased from *BioRad* and *Sigma*. Standard bovine thrombin and fibrinogen were from *Sigma*. Sephadex G-75 and Benzamidine-Sepharose 6B, were from *Sigma-Aldrich* Co. The diagnostic kit for fibrinogen was from *Wiener Laboratories* S.A.I.C (Rosario, Argentina) and human fibrinogen type I for the specific cleavage study was from *Sigma*. All other reagents were laboratory grade.

Purification of thrombin-like enzyme

Purification of the thrombin-like enzyme was performed employing gel filtration and affinity chromatography[9] with the modification introduced by Camillo *et al*[16]. In both cases the second step in the purification was performed by affinity chromatography in a Benzamidine-Sepharose 6B column, where they had used a buffer containing 50 mM Tris, 250 mM NaCl and 150 mM benzamidine at pH 9.0, to elute the thrombin-like enzyme from the column, we used a 0.1M sodium acetate buffer, pH 5.0, containing 0.15M NaCl. The elution was monitored at 280 nm and enzymatic essays were carried out as described below.

Analytical procedures

Estimation of protein

The protein determination of venom fractions was determined by measuring the absorbance at 280 nm in a UV-visible *CamSpec M 330* spectrophotometer with an optical path of 1 cm, based upon the assumption that the absorbance of the 1 mg/ml of protein of venom fraction was 1.183, the same as that of crude venom.

SDS-PAGE

Purified gyroxin and crude venom samples were dissolved in phosphate buffer, pH 7.00, containing 1% (w/v) SDS, 0.05% (w/v) 2-mercaptoethanol, 5% urea and 0.005% bromophenol blue. Samples were heated for 3 min at 100°C an applied on top of 12% polyacrilamide slab gel[17]. Protein bands were revealed by Coomassie Brilliant Blue R-250 staining procedure. Molecular weight markers run in parallel were phosphorilase B, bovine serum albumin, ovoalbumin, carbonic anhydrase, soybean trypsin inhibitor and lyzosyme (*Bio-Rad*).

Specific cleavage of fibrinogen and fibrin by crude venom and purified enzyme was performed using 12% polyacrilamide slab gel[17]. Samples were dissolved in 35 mM phosphate buffer, pH 7.00, containing 2% (w/v) SDS, 2% (w/v) 2-mercaptoethanol, 5% urea and 0.005% bromophenol blue and heated for 3 min at 100 °C. Molecular weight markers run in parallel were bovine serum albumin, ovoalbumin, carbonic anhydrase, trypsinogen (PMSF treated), trypsin inhibitor and α-lactalbumin (*Sigma*).

Enzymatic and toxic essays

Thrombin-like activity

Thrombin-like activity was assayed by recording clotting time for 0.2 ml of diluted bovine plasma (3:1 with 20 mM Tris HCl buffer, pH 8.0) with 0.1 ml of either crude venom or chromatographied venom fractions at 37 °C, according to the method described by Raw *et al.*[5] with minimal changes in the quantities and reagents. The clotting times obtained were transformed into NIH units by means of a standard thrombin-fibrinogen curve constructed according to Lundblad[18].

Gyroxin syndrome test

It was performed using swiss males and female mice with body weight 25-30 g inoculated by i.p. injection with different doses of purified protein in a rank of 0.02 to 3.2 µg/g body weight and observed during four hours.

Effects of gyroxin and C. d. terrificus *venom on the mice plasma fibrinogen*

The *in vivo* test was performed using eight groups of mice with different exposure time and one control group, each one composite by four male mice weighing 18-22 g, which were i.p injected with 0.5 µg/g body weight of purified thrombin-like enzyme. The different groups were:

Group 1: 30 minutes; group 2: 60 minutes; group 3: 3 hours; group 4: 6 hours; group 5: 8 hours; group 6: 10 hours and group 7: 24 hours. After the time of exposure, the animals were anesthetized with chloral hydrate (300 mg/kg body weight) and two ml of blood were collected through the cava vein. The blood (1.8 ml) was anticoagulated with 0.2 ml of trisodium citrate (0.129M) in a blood collection tube. Platelet-poor plasma was obtained after centrifugation at 2500 rpm at room temperature for 15 minutes. The fibrinogen content was determined (see fibrinogen determination). Each sample was measured by triplicate and the mean value of fibrinogen concentration was calculated. The control group (group 8) was not inoculated with gyroxin; only blood samples were collected to determine standard plasma fibrinogen levels.

The same procedure was carried out with crude venom, so the groups with different time of exposure assayed and the dose were the same. In this experience, two hours after venom inoculation, the animals were treated with anticrototalic serum to avoid mice death. This latter may happen two hours after venom inoculation.

Plasmatic fibrinogen determination. Plasmatic fibrinogen level was determined by the diagnostic kit (*Wiener*, fibrinogen) based on the other method[19], the procedure is a clotting rate method with the addition of a very high bovine thrombin concentration to a 10-fold diluted plasma and renders the logarithm of the clotting time directly proportional to the logarithm of fibrinogen concentration. Determinations and calibration curve were performed according to the manual of the manufacturer.

Specific hydrolysis of fibrinogen

Specific cleavage of fibrinogen by the crude venom and the isolated enzyme was shown by electrophoresis on 12% polyacrylamide gels. One hundred µl of human fibrinogen at a concentration of 5 mg/ml in 0.1 M Tris-HCl buffer (pH 8) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3, was added to 100 µl of the isolated thrombin-like enzyme or crude venom at a concentration of 250 µg/ml in the buffer previously mentioned, and incubated at 37 °C. At various time intervals, aliquots of 30 µl were withdrawn from the digestion mixture and the reaction was stopped by addition of 30 µl of denaturing solution (35 mM phosphate buffer, pH 7.00, containing 2% SDS, 2% 2-mer-captoethanol, 5% urea and 0.005% bromophenol blue). The samples were denatured and reduced by boiling for 3 min prior the run on SDS-polyacrilamide gel[20].

Degradation of fibrin

Fibrinolytic activity. A modified plaque assays[21] was used to measure fibrinolytic activity of the venom and the thrombin-like enzyme. Citrated sheep plasma was diluted 1:2 with a buffer containing 50 mM Tris, pH 7.5. Twenty-two milliliters were been transferred to plastic dishes (13 x 8 cm), 2.2 ml of 0.25 M $CaCl_2$ and 132 µl human thrombin (*Sigma Chemical*) were added to produce a clot. After 60 min of incubation at 37 °C wells of 2 mm diameter were made in the clot and 10 µl of venom solutions (ranging from 0.156-40 mg/ml) or thrombin-like enzyme (ranging from 0.125-5.0 mg/ml) were applied to each well. Incubations were carried out at 37 °C for 18 h, after which the diameter of the fibrinolytic halos was measured. The minimum fibrinolytic concentration (MFC) was defined as the concentration of venom (final, mg/ml) that induced a fibrinolytic halo of 12 mm diameter. The assay was performed three times.

Fibrin degradation products. Fibrin hydrolysis was demonstrated by SDS-PAGE using 12% polyacrilamide gels. Two hundred and fifty microliters of thrombin 10 units/ml was added to 250 µl of a 10 mg/ml fibrinogen solution in 0.1 M Tris-HCl buffer (pH 8) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3. The fibrin clot was allowed to form for 1 h at room temperature. After that, 500 µl of thrombin-like enzyme or crude venom (250 µg/ml) was added to the clot and incubated at 37 °C. At various time intervals, aliquots (30 µl) of incubation mixture was withdrawn and added to 30 µl of denaturing solution (35 mM phosphate buffer, pH 7.00, containing 2% SDS, 2% 2-mercap-toethanol, 5% urea and 0.005% bromophenol blue). The samples were reduced and denatured in a boiling water bath for 3 min and run on SDS-polyacrilamide gels[20].

Light microscopy for intravascular coagulation detection

In order to have a histological assessment of *in vivo* clot formation ability by the isolated thrombin-like enzyme, groups of four mice were injected i.m. in the right gastrocnemius with 0.1 ml of solution containing the enzyme isolated (9 µg). Four mice were used as control samples, receiving each of them 0.1 ml of phosphate buffered saline solution (pH 7.2). After 60 and 120 minutes of injection mice were anesthetized with cloral hydrate i.p. 300 mg/kg. Mice were sacrificed by cervical dislocation and samples of cardiac muscle, lung and liver were taken and fixed in Bouin solution for 24-48 h. Thereafter, the samples were dehydrated in a graded alcohol series and embedded in paraffin. Sections 10 µm thick were stained with hematoxylin-eosin (HE).

Results

Purification

The results from a typical purification of gyroxin are shown in Table 1. The venom was purified in a two-step procedure. The elution profile from the G-75 chromatography is shown in Fig. 1 and that of the Benzamidine-Sepharose 6B chromatography in Fig. 2. After procedure the enzyme obtained had 34-fold purification factor and a 12.1% in activity yield. Starting with 250 mg of venom, 0.9 mg of pure thrombin-like enzyme was obtained. As shown in Table 1, the Sephadex G-75 column was an efficient step in this procedure, giving 5-fold purification. The enzyme was eluted with glycine buffer, 20 mM, pH=1.9, containing 150 mM of sodium chloride. As shown in Fig. 1 most of the thrombin-like activity eluted in the higher molecular weight region. Starting with 250 mg of venom, 23 mg of this protein was obtained in this step. Those fractions containing thrombin-like activity were pooled and applied

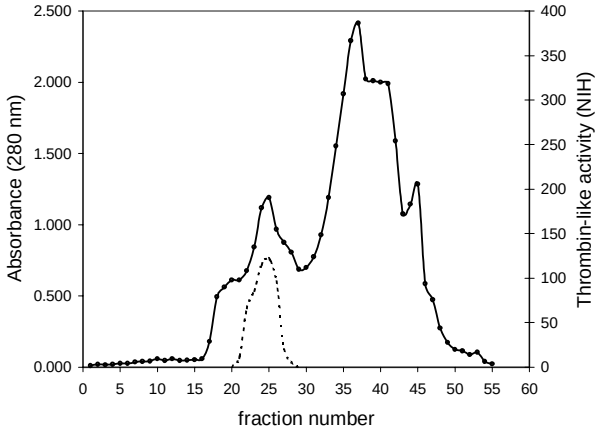

Fig. 1.– Elution profile of *Crotalus durissus terrificus* venom fractionated on Sephadex G-75. Venom (50 mg/ml, 5 ml) was applied to a column (2.0 x 75 cm) of Sephadex pre-equilibrated and eluted with glycine buffer 20 mM, 150 mM NaCl, pH 1.9. Five ml fractions were collected at a flow rate of 12 ml/h and assayed for absorbance at 280 nm (—•—) and thrombin-like activity (- - - -).

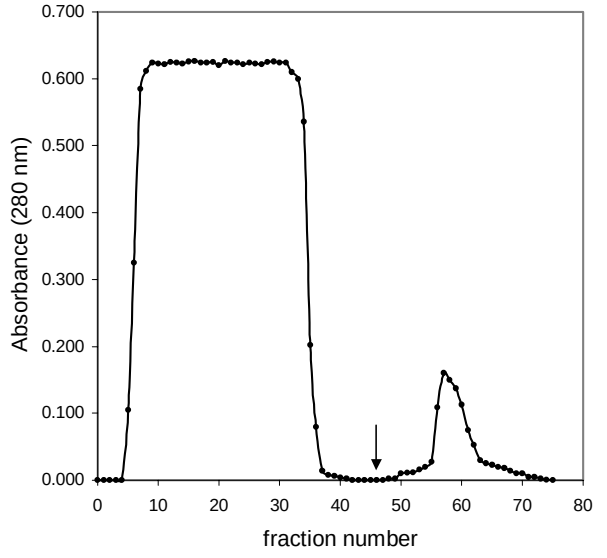

Fig. 2.– Benzamidine-Sepharose chromatography of pooled fractions from the Sephadex G-75 gel filtration column. Elution profile of Benzamidine-Sepharose 6B (1.0 x 6.0 cm) equilibrated and run with 50 mM Tris, 400 mM NaCl, pH 9.0. The line represents absorbance at 280 nm. The pooled sample from the G-75 step (35 ml) was pH and ionic adjusted as described by Alexander *et al*[9]. The sample was loaded and then the column was washed with running buffer until the absorbance at 280 nm returned to baseline. At the point indicated by the arrow the running buffer was changed to sodium acetate buffer, 100 mM, pH = 5.0, containing 150mM of sodium chloride. One protein peak eluted contained the thrombin-like activity.

to the Benzamidine-Sepharose 6 B affinity column (step 2). There was one protein retained by the benzamidine

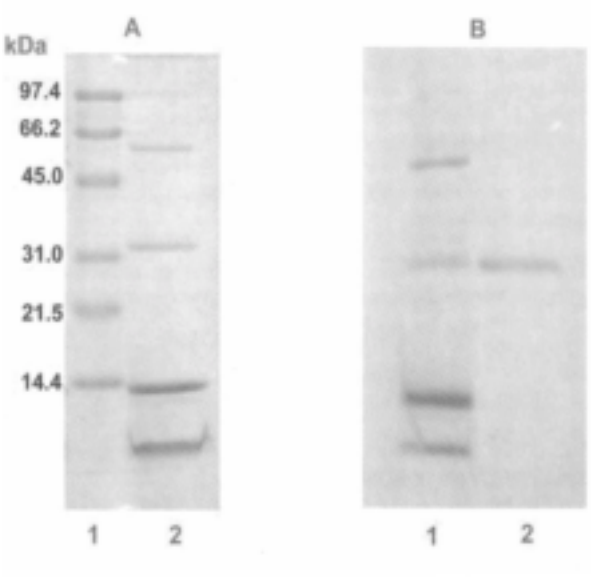

Fig 3.– Electrophoretic profiles of gyroxin and *Crotalus durissus terrificus* venom. A: SDS-polyacrilamide 12% gel electrophoresis of: *Crotalus durissus terrificus* crude venom (lane 2), molecular weight markers (lane 1). B: SDS-polyacrilamide 12% gel electrophoresis of *Crotalus durissus terrificus* crude venom (lane 1), purified thrombin-like (gyroxin) (lane 2).

column (Fig. 2) and eluted with sodium acetate buffer, 100 mM, pH= 5.0, containing 150 mM of sodium chloride which exhibited thrombin-like activity. This step resulted in 6-fold purification of the enzyme; besides, it was essential for obtaining a protein of higher homogeneity. SDS-PAGE attributed a value of 33 kDa for the enzyme isolated after affinity chromatography, this value is similar to those obtained by other authors (34 kDa[9], 36 kDa[16]) (Fig. 3). The enzyme isolated has a thrombin-like activity as demonstrated by its ability to clot bovine plasma, exhibiting a higher activity than the one corresponding to the crude venom (Table 1)

Gyroxin syndrome test

The animals injected with a low doses (0.02 to 0.25 µg/g body weight) showed in the first minutes a brief period of hyper-excitation with running and grooming behavior, after that between 10 min to 20 min was observed a sudden stretching of body and posterior limbs accompanied by opisthotonos with decrease in the time of righting reflex compared with control animals. All symptoms disappeared after half past one hour and the animal behavior returned to normal. On the other hand, mice injected with higher doses (0.34 to 0.75 µg/g body weight) developed

TABLE 1.– *Purification of gyroxin from* Crotalus durissus terrificus *venom*

	Protein (mg)	Thrombin (NIH units)	Specific act. (units/mg)	Recovery* (%)
Venom	250	1120	4.48	100
Step 1: Pool G-75	23.0	558	24.2	49.8
Step 2:				
Benzamidine-Sepharose				
Non-retained	22.1	20	0.90	1.6
Retained (gyroxin)	0.9	135	150	12.1

** The percent recovery is based on the thrombin activity*

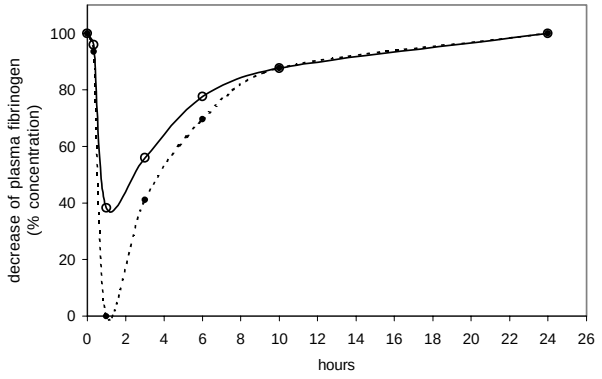

Fig. 4.– Loss of mouse plasma fibrinogen after intravenous injection of thrombin-like enzyme isolated (- -•- -) and *C. d. terrificus* venom (—o—). Values are means of three determinations for each mice group (n=4).

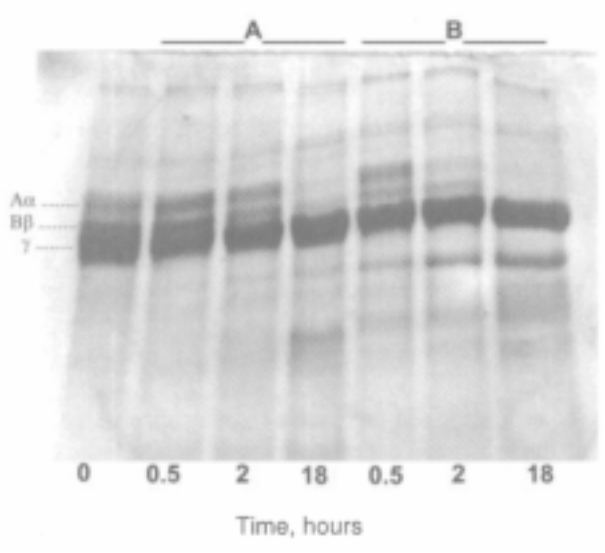

Fig. 5.– Fibrinogen degradation by the venom and the isolated enzyme. First lane (left) fibrinogen control incubated without enzyme for 10 min. Fibrinogen incubated for the specified times with venom (lanes A). Fibrinogen incubated for the specified times with the isolates enzyme (lanes B).

symptoms such as immobility following by tachypnea with apparently breathing problem and stretching of posterior limbs. Not one the doses were lethal and all animals returned to normal behavior. All results confirmed that the isolated enzyme is gyroxin.

Effect of gyroxin and C. d. terrificus venom on the mice plasma fibrinogen

The effect of *C. d. terrificus* venom and purified thrombin-like enzyme were examined *in vivo*. The experiments showed that the mice plasma fibrinogen decreases rapidly during the first hour after the thrombin-like enzyme injection, until non detected values, then reaching its normal level 10 h after injection; whereas the crude venom, when injected in vivo, results in a 60% decrease of the mice plasma fibrinogen, reaching its normal level after the same period of time (Fig. 4) It is important remark again that the anticrotalic serum did not affect the evolution of plasmatic fibrinogen levels, since this latter was registered when the antivenom was not yet given to the mice.

Degradation of fibrinogen

Both crude venom and the thrombin-like enzyme exhibited fibrinogenolytic activity. SDS-PAGE analysis showed the thrombin-like enzyme isolated to be an Aα Bβ fibrinogenase with the Aα chains being degraded within 2 hours followed by a slower degradation of the Bβ chains (Fig. 5). The enzyme appeared to have no effect on the γ chain after of 18 h of hydrolysis. Fibrinogen degradation products of different molecular weight early appeared and increased during hydrolysis, indicating extensive hydrolysis of the fibrinogen.

The crude venom exhibited a weak Aα Bβ fibrinogenolytic activity. Partial hydrolysis of Aα and Bβ chains

was only observed after 2 h, and the almost total degradation was after 18 h. At these times, fibrinogen degradation products can be observed; some of them exhibit different molecular weights from those produced by the isolated thrombin-like enzyme (Figure 5).

Degradation of fibrin

Fibrinolytic activity and cleavage specific of fibrin

The isolated enzyme showed a strong fibrinolytic activity when it was added to a fibrin clot by the plaque method. The halos were evaluated after 18 h of incubation and were proportional to the amount of enzyme; the obtained linear relationship (r = 0.997) let us determine the MFC. The concentration of purified thrombin-like enzyme that induced a fibrinolytic halo of 12 mm diameter was 2.30 mg/ml.

The crude venom showed a weak fibrinolytic activity when it was added to a fibrin clot by the plaque method. Halos of 12 mm diameter were not observed even when high doses of 40 mg/ml were used. For that reason, the MFC for the whole venom could not be determined.

The degradation of fibrin was examined by SDS-PAGE to determinate which subunit of this protein was affected by the crude venom and by the isolated enzyme. The hydrolysis of fibrin by the crude venom resulted in the degradation of the α-chains, while the β-chains, the γ-chain and the dimmer γ-γ appeared unaffected throughout the incubation period examined (Fig. 6). Degradation products of high molecular weight (40 - 45 kDa), of molecular masses between 20 and 24 kDa and of molecular weight minor than 14 kDa were observed within the first two hours of hydrolysis. After 18 hours, these bands appeared more intensified.

A similar experiment performed with the purified enzyme indicated that it was able to hydrolyze the α- and β-chains of fibrin whereas it appeared to have no effect on the γ chain and dimmer γ-γ even after 18 h incubation (Fig. 6). Partial hydrolysis of α- and β-chains was observed after 2 h. They were completely degraded after 18 h. Degradation products of molecular weight around 33 kDa appeared after 2 h incubation. After 18 h, a new band appeared with molecular mass around 22 kDa. Therefore, fibrin degradation products obtained with the iso-

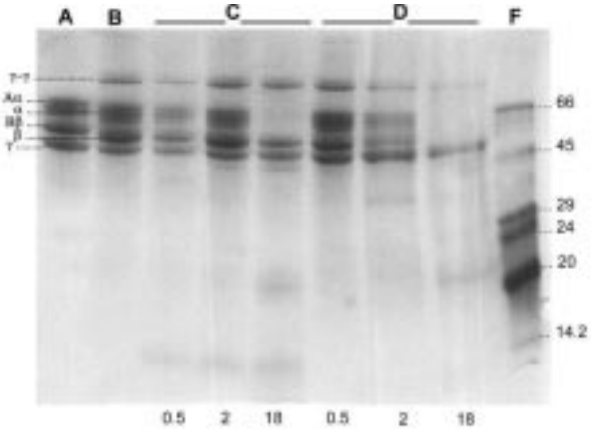

Fig. 6.– Fibrin degradation by the venom and the isolated enzyme. Fibrinogen control incubated without enzyme for 10 min (lane A). Fibrin control incubated without enzyme for 10 min (lane B). Fibrin incubated for the specified times with venom the isolates enzyme (lanes C). Fibrin incubated for the specified times with the isolated enzyme (lanes D). Molecular weight markers (lane F).

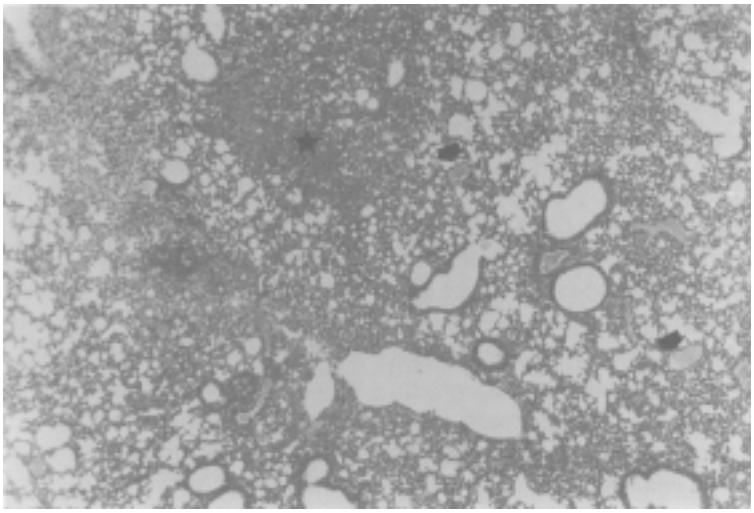

Fig. 7.– Histological cutting of lung of mouse inoculated with 9 µg of gyroxin. Time of exposure: 2 h. Pulmonar congestion, edema and intralveolar hemorrhage (star), accompanied by thrombosis in blood vessels (arrow), were observed (40xHE).

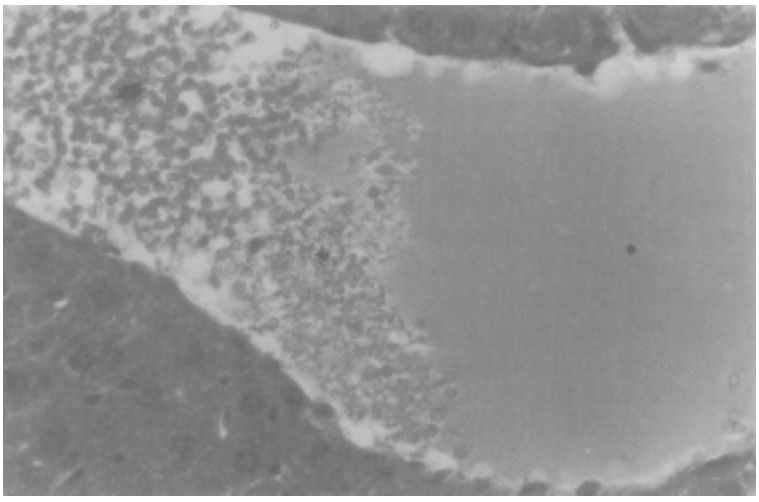

Fig. 8.– Histological cutting of liver of mouse inoculated with 9 µg of gyroxin. Time of exposure: 2 hs. Vascular thrombosis characterized by abundant fibrin (asterisk) was observed. Platelets (star) and blood red cells (arrow) surround the clot (400xHE).

lated gyroxin were no similar to those obtained with the crude venom.

Histological assay

After 1 hour of gyroxin inoculation, congestion of small coronary blood vessels, arterioles and capillaries among the muscular fibers were observed. After two hours, intravascular coagulation was detected.

In cuttings of lung, after 1 hour of enzyme injection, congestion of alveolar septums and intravascular thrombosis were observed. After two hours, lesions were more intense (Fig. 7). In cuttings of liver, lesions compatible with intravascular coagulation were observed in central veins (Fig. 8).

On the whole, in every studied tissues it was verified that the process begins with the congestion of small blood vessels, arterioles and capillaries. After that, intravascular coagulation, characterized by abundant fibrin with platelets and blood red cells joined to the clot, is manifested. It demonstrates that the isolated enzyme possesses *in vivo* coagulant activity.

Discussion

In this paper a thrombin-like enzyme was isolated, through a modified technique as described by the literature[9], then this enzyme was used to study its effects on haemostatic system in mice. Regarding to the methodology employed to isolate the enzyme, we observed that the markedly acidic pH in the first step, allowed to obtain the enzyme among the first fractions of eluted proteins, favoring its separation from the major proteins contained in the venom (crotoxine complex), without affecting the activity of the enzyme[9].

The modification introduced by Camillo *et al.*[16] in the second step let us an easy elution of the protein without sample consumption, at the same time it allowed to elute the enzyme in its biologically active form, being the clotting activity of the isolated fraction much more intense than the one corresponding to the crude venom.

Low recovery percentage of the isolated enzyme (0.4% of the total protein present in the original venom sample) and the reduced clotting activity of the whole venom could indicate a low concentration of thrombin-like enzyme in the venom of the rattlesnake that inhabits the northeast region of Argentina. It is important to clear up that Seki *et al.*[22] obtained a higher output (0.8-1%) when they purified the enzyme presents in the venom extracted from *C. d. terrificus* specimens coming from Santiago del Estero (central region of Argentina). This different output with regard to ours, could be explained considering that venoms, obtained from specimens of different regions, have different compositions, as described Cavinato *et al.*[12]. Also, the used methodology in that case, twenty years ago, could not have let an efficient purification of the enzyme.

The purified thrombin-like enzyme induced changes in the animal behavior, eliciting a sintomatology that coincides with the “gyroxin syndrome”, described by other authors[9, 22]. It confirms that the isolated protein is gyroxin. Symptoms varied according to the dose inoculated presenting a more complete syndrome with the smallest dose used. It points out a non-lineal relationship between dose and effect as showed in other studies with this enzyme[22].

Gyroxin, with a higher coagulant activity than the one corresponding to the crude venom, caused plasmatic fi-

brinogen consumption in a way more intense than crude venom. In the first hours, the fibrinogen consumption after injection was quick, whereas with crude venom the consumption was lower. Fibrinogen levels were normal after ten hours with either thrombin-like enzyme or crude venom.

The purified enzyme showed *in vitro* and *in vivo* plasma clotting activity. In that sense, intravascular clots observed in histological cuttings confirmed this ability. On the other hand, *in vitro* studies demonstrated that the isolated enzyme was capable to degrade fibrinogen and fibrin. Although Raw *et al*.[5] observed that the thrombin-like isolated from *Crotalus durissus terrificus* only hydrolyze the Aα chains of fibrinogen, we demonstrated that our enzyme degrades the Bβ chains besides the Aα ones. These results agreed with those reported by Markland Jr and Damus[4], who described that the thrombin-like isolated from *Crotalus durissus adamanteus* hydrolyzes the Aα chains of fibrinogen and also slowly hydrolyzes the Bβ chains. Besides, we demonstrated that the thrombin-like enzyme from *Crotalus durissus terrificus* exhibited hydrolytic activity on fibrin: α-β-chains were partially hydrolyzed two hours after incubation and completely hydrolyzed after 18 hours.

These results let conclude that probably the enzyme *in vivo*, besides consuming fibrinogen by coagulation, directly hydrolyses fibrinogen. It is also possible the enzyme degrades fibrin clots as it was demonstrated *in vitro*. However, the observation of clots in histological cuttings obtained two hours after enzyme injection demonstrates an initial action of the enzyme, which then metabolizes allowing the restatement of plasma fibrinogen level, being the liver, in physiological conditions, the organ responsible of doing this latter.

Regarding to the crude venom, it exhibited weak Aα Bβ fibrinogenolytic activity and α fibrinolytic activity throughout the incubation period examined. The plaque method showed small halos, even to high doses, so the MFC for the whole venom from *Crotalus durissus terrificus* could not be determined. Considering that other crotaline venoms show fibrinolytic activity (MFC: 2.56 mg/ml for *Crotalus durissus durissus,* 1.99 mg/ml for *Bothrops asper*$_{Pacific}$; 3.59 mg/ml for *Bothrops asper*$_{Atlantic}$) [23] as our isolated enzyme from *Crotalus durissus terrificus* show also (2.30 mg/ml), it is probable that this very low activity exhibited by the venom is due to the low percentage of the enzyme in the crude venom. It is also possible the presence of other proteolytic enzymes in the crude venom since fibrinogen and fibrin degradation products, different from those obtained with the enzyme isolated, were found.

Results here obtained, show the ability of the isolated thrombin-like enzyme of consuming fibrinogen by coagulation, and probably by hydrolysis too. They also demonstrate the rapidity of fibrinogen reinstatement, even with a high amount of the enzyme. Such observations, added to the low levels of the coagulant activity detected in the venom of the rattlesnake that inhabits the northeast region of Argentina, could explain the low incidence of the clotting dysfunction present in the accidents caused by these snakes. However, it is important to amplify the knowledge about the physiopathology of the intoxication by rattlesnake in order to apply an efficient treatment, especially in patients with hepatic or clotting diseases, where the replacement of the fibrinogen could be delayed, even inhibited, leading to serious alterations in the haemostatic system that could complicate the recovery of the victim.

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To define genes by the diseases they cause is about as absurd as defining organs of the body by the diseases they get: livers are there to cause cirrhosis, hearts to cause heart attacks and brains to cause strokes. It is a measure, not of our knowledge but of our ignorance that this is the way the genome catalogues read. It is literally true that the only thing we know about some genes is that their malfunction causes a particular disease. . . But many diseases are caused by the fact that the gene is missing altogether. In the rest of us, the gene is a positive, not a negative force. The sufferers have the mutation, not the gene.

Definir los genes por las enfermedades que causan es casi tan absurdo como definir los órganos del cuerpo por las enfermedades que tienen: el hígado está ahí para causar cirrosis, el corazón para causar infartos y el cerebro para causar apoplejías. El hecho de que los catálogos del genoma se interpreten así no es una medida de nuestro conocimiento sino de nuestra ignorancia. Es literalmente cierto que lo único que sabemos de algunos genes es que su mal funcionamiento produce una enfermedad determinada. . . Pero muchas enfermedades se deben a la completa ausencia del gen. En el resto de nosotros, el gen es una fuerza positiva, no negativa. Los enfermos tienen la mutación, no el gen.

Matt Ridley

Genome: the autobiography of a species in 23 chapters. New York: Harper Collins, 1999, p 54
(Genoma: la autobiografía de una especie en 23 capitulos. Traducción de Inés Cifuentes. Madrid: Grupo Santillana de Ediciones S.A., 2000, p 69)