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ISOLATION OF A MYOTOXIN FROM *BOTHROPS ASPER* VENOM: PARTIAL CHARACTERIZATION AND ACTION ON SKELETAL MUSCLE

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J. M. GUTIÉRREZ, C. L. OWNBY and G. V. ODELL. Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. *Toxicon* 22, 115–128, 1984. — A myotoxic phospholipase has been isolated from *Bothrops asper* venom by ion-exchange chromatography on CM-Sephadex followed by gel filtration on Sephadex G-75. The toxin is a basic polypeptide with an estimated molecular weight of 10,700. It has both phospholipase A and indirect hemolytic activities, but is devoid of proteolytic, direct hemolytic and hemorrhagic effects. When injected i.m. into mice the toxin induces a rapid increase in plasma creatine kinase levels and a series of degenerative events in skeletal muscle which lead to myonecrosis. The toxin induces an increase in intracellular calcium levels and is able to hydrolyze muscle phospholipids *in vivo*. Pretreatment with the calcium antagonist verapamil failed to prevent the myotoxic activity. It is proposed that *B. asper* myotoxin causes cell injury by disrupting the integrity of skeletal muscle plasma membrane and that myotoxicity is at least partially due to the phospholipase A activity of the toxin.

INTRODUCTION

FROM a public health point of view *Bothrops asper* is the most important snake in Costa Rica (JIMÉNEZ and GARCÍA, 1969). Its venom induces a complex and severe local tissue damage, characterized by edema, hemorrhage and myonecrosis (PICADO, 1931; JIMÉNEZ and GARCÍA, 1969). Experimentally, these effects have been studied in mice at both the light and electron microscopic levels (TU and HOMMA, 1970; GUTIÉRREZ *et al.*, 1980; ARROYO and GUTIÉRREZ, 1981), as well as by quantitating the plasma concentration of creatine kinase (GUTIÉRREZ *et al.*, 1980). Myonecrosis induced by this venom is particularly important, since experimental studies have shown that the polyvalent antivenom used in Costa Rica is only partially effective in neutralizing myotoxicity (GUTIÉRREZ *et al.*, 1981).

To provide a better understanding of this pathological phenomenon, and to improve antiserum treatment of *B. asper* snakebite cases, a myotoxin has been isolated from this venom. The biochemical properties of the toxin and its physiologic effects on skeletal muscle are reported in the present study.

MATERIALS AND METHODS

Venom and chemicals

B. asper venom was generously provided by Dr. Luis Cerdas, Instituto Clodomiro Picado, Universidad de Costa Rica, San José, Costa Rica. The venom was obtained from adult specimens from the Atlantic region of

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Costa Rica. After collection, the venom was centrifuged at low speed, freeze-dried and stored at −70°C. All other drugs and chemicals were obtained from the usual commercial sources and were of the highest grade available.

Isolation of the myotoxin

Samples of 200 mg of venom were dissolved in 2 ml of 0.05 M Tris, 0.1 M KCl (pH 7.0) buffer and chromatographed on a column of CM-Sephadex C-25 (150 × 2 cm) which had been equilibrated with 0.05 M Tris, 0.1 M KCl (pH 7.0) buffer. The column was eluted with 200 ml of the same buffer and then a linear salt gradient of 0.05 – 0.75 M KCl was performed, in a total volume of 500 ml. Fractions of 3.5 ml were collected and the absorbance at 280 nm of each tube was recorded. The different fractions were pooled, dialyzed against distilled water and freeze-dried. Once checked for biological activity, fraction 5 was dissolved in 2 ml of 0.05 M Tris, 0.1 M KCl (pH 7.0) buffer and applied to a Sephadex G-75 column (100 × 1.5 cm) that had been previously equilibrated with the same buffer. Three milliliter fractions were collected, their absorbances recorded at 280 nm and the peaks pooled, dialyzed against distilled water and freeze-dried.

Homogeneity

Polyacrylamide disc gel electrophoresis was performed at pH 4.3, according to the method of REISFELD *et al.* (1962). The electrophoretic run was performed at a current of 4 mA/tube. The gels were then stained with 0.5% aniline blue black in 7% acetic acid for 30 min and were destained in 7% acetic acid.

Immunodiffusion

The Ouchterlony double-diffusion technique (CLAUSEN, 1969) was performed in 1% agarose gels for 18 hr at room temperature. The following antigens were used: *B. asper* myotoxin; *B. asper* crude venom; *B. atrox* crude venom; myotoxin *a* isolated from the venom of *Crotalus viridis viridis* according to the procedure of OWNBY *et al.* (1979). Antigen concentration was 1 mg/ml. The polyvalent antivenom from the Instituto Clodomiro Picado, Costa Rica, produced in horses immunized with a mixture of venoms of *Bothrops asper, Crotalus durissus durissus* and *Lachesis muta* (BOLAÑOS and CERDAS, 1978), was used. In some experiments the antimyotoxin *a* serum produced in this laboratory (OWNBY *et al.*, 1979) was also used.

Molecular weight estimation

Molecular weight was estimated by gel filtration in Sephadex G-75 in a column of 100 × 1.5 cm, using 0.05 M Tris, 0.1 M KCl (pH 7.0) as elution buffer. The following standards were used: bovine serum albumin; ovalbumin; chymotrypsinogen A; cytochrome c.

Myotoxic activity

Myotoxic activity was evaluated by histology and quantitation of the plasma levels of creatine kinase. Groups of 4 mice (20 – 24 g) were injected i.m. in the thigh with the toxin (2.5 μg/g) and with the crude venom (2.5 μg/g). At intervals after inoculation (30 min, 1 hr, 3 hr, 6 hr, 12 hr, 24 hr and 48 hr) a blood sample was taken by cutting the tip of the tail. Blood was collected in heparinized capillary tubes and plasma was obtained after centrifugation. Immediately, mice were killed and a muscle sample was obtained from the injected thigh, cut into small pieces and immersed in 2.5% glutaraldehyde for 2 hr. Tissue was processed and observed according to the procedure of OWNBY *et al.* (1976). Plasma creatine kinase was estimated according to Sigma Technical Bulletin 520 (St. Louis, Missouri) and was expressed in units/ml; one unit of creatine kinase results in the phosphorylation of 1 nmol of creatine per min at 25°C.

Hemorrhage

Mice (20 – 24 g) were injected i.m. in the thigh with the toxin (2.5 μg/g). At different time intervals they were killed, the skin of the thigh removed and the presence of hemorrhage assessed by ocular inspection. Tissue was processed for histology as described above and examined for the presence of microscopic hemorrhage.

Hemolytic activity

Both direct and indirect hemolytic activities were evaluated by the method of JENG *et al.* (1978). Indirect hemolytic assay was performed in tubes containing 10 μg of the toxin, 0.3 mg of egg yolk phosphatidylcholine, 0.6 ml of a 2.5% human erythrocyte suspension and 50 μl of 10 mM $CaCl_2$ solution. The volume was made up to 3.0 ml with pH 7.5 physiologic saline solution. The incubation mixture for the direct hemolytic assay contained the same reagents, with the exception of phosphatidylcholine. The toxin was excluded from the blank tube. In order to have a 100% hemolysis value, erythrocytes were incubated with distilled water. Mixtures were incubated at 37°C for 30 min, then centrifuged at low speed in a clinical centrifuge for 5 min and the absorbances read at 540 nm.

Phospholipase activity in vitro

The method of HABERMANN and HARDT (1972) was followed. A suspension of egg yolk was incorporated into 0.6% agarose gels. Cylindrical holes were punched and emptied by suction. Each hole was filled with 10 μl of toxin solution and the plates were incubated at 50°C for 20 hr. Phospholipase A activity was indicated by clearing of the egg yolk in the plate. In some experiments erythrocytes were included in the gel and the diameter of the

hemolytic halo was measured in order to increase the specificity of the assay.

Proteolytic activity

Hide powder azure (Sigma Chemical Co., St. Louis, MO) was used as substrate for proteolytic activity, according to the method of RINDERKNECHT *et al.* (1968).

Calcium studies

In order to follow changes in cytoplasmic calcium levels of skeletal muscle, a modification of the technique of MURPHY *et al.* (1980) was developed using the metallochromic calcium indicator Arsenazo III (SCARPA *et al.*, 1978). Groups of 4 mice were injected i.m. in the thigh with the myotoxin (5 μg/g). At three different time intervals (1 hr, 3 hr and 6 hr) mice were killed and a sample of muscle was obtained from the thigh and blot dried on filter paper. The tissue (50 mg) was transferred to a vial containing Arsenazo III (0.1 mM), digitonin (200 μg/ml), NaCl (140 mM), KCl (5 mM) and imidazole (10 mM) (pH 7.0) in a total volume of 2.0 ml. After exactly 1 min, a 1 ml aliquot of the supernatant was transferred to another vial. The absorbance of this solution was recorded at 675 and 685 nm using a dual wavelength program in a Beckman DU-8 spectrophotometer. Calcium levels are directly proportional to the differential absorbance of Arsenazo III at 675 – 685 nm. Due to the presence of digitonin, calcium that is released to the medium is mainly cytoplasmic (MURPHY *et al.*, 1980). Therefore, this method follows changes in cytoplasmic calcium instead of total calcium. Groups of 4 mice were injected i.m. with 0.1 ml of physiologic saline solution as controls. At the same time intervals, pieces of muscle were taken and treated under the same experimental conditions as described above.

Neutralization of myotoxicity by verapamil

Verapamil is a calcium antagonist which acts on the voltage-sensitive slow calcium channels. To investigate whether the calcium influx induced by the toxin takes place through these channels, several experiments were performed. Groups of 4 mice (20 – 24 g) were pretreated i.m. in the thigh with verapamil at doses of 2.5, 5 and 10 μg/g. Five minutes later, the toxin (2.5 μg/g) was injected i.m. at the same site. In another experiment, verapamil (5 μg/g) and toxin (2.5 μg/g) were inoculated i.m. simultaneously. Three hours after injection blood samples were taken by cutting the tip of the tail and the plasma levels of creatine kinase were quantitated as previously described. This time was selected since it is when the highest creatine kinase levels are observed after toxin inoculation. Twenty-four hours after injection, mice were killed and a muscle sample (primarily sartorius) was obtained from the injected thigh and placed in glutaraldehyde fixative solution. Tissue was cut in order to facilitate orientation for cross-sections of the muscle and was processed for histological observation as described above. Once stained, muscle cells were counted using a Zeiss light microscope equipped with a Zeiss MOP 3 Image Analyzer. A total of 5 slides were counted from each mouse. Myonecrosis was expressed as myonecrosis index (number of necrotic cells compared to total number of cells) (OWNBY *et al.*, 1983).

Hydrolysis of phospholipids in vivo

Groups of 4 mice were injected i.m. in the thigh with myotoxin (5 μg/g). At 30 min, 3 hr and 6 hr mice were killed and a piece of muscle removed from the thigh. It was immediately homogenized in a glass homogenizer with 200 μl distilled water. Then, 1.5 ml of methanol/ chloroform (2:1) was added and the suspension shaken and centrifuged for 10 min at 1200 *g*. The supernatant was collected, transferred to another tube and 1.9 ml of methanol/chloroform/water (2:1:0.8) was added to the pellet. The suspension was shaken and centrifuged for 10 min at 1200 *g*. The second supernatant was added to the first one. Then they were diluted with 1.0 ml chloroform and 1.0 ml distilled water and the mixture centrifuged at 2500 *g* for 5 min. The chloroform layer was collected with a Pasteur pipette and the solvent was evaporated. The resultant sediment was resuspended with 50 μl of chloroform and aliquots of 10 or 15 μl were applied to precoated TLC silica gel 60F-254 plates (EM). Plates were run in chloroform/methanol/acetic acid/water (54:25:8:4). Pure phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine (Sigma, St. Louis, MO) were also applied. At the end of the run, plates were dried, sprayed with concentrated sulfuric acid – 30 % formaldehyde (97:3 by volume) and heated at 90°C for 20 min in order to visualize the spots.

RESULTS

Isolation

Figure 1 shows the profile of the ion exchange chromatographic (CM-Sephadex) separation of *B. asper* venom. Fractions 1, 4 and 5 showed myotoxic activity when tested in mice, whereas all of the hemorrhagic activity was located in fractions 1 and 2. When submitted to polyacrylamide disc gel electrophoresis, fraction 5 showed a single band. However, in order to assure purity, this fraction was submitted to gel filtration with Sephadex G-75. A single symmetrical peak was obtained which was pure according to

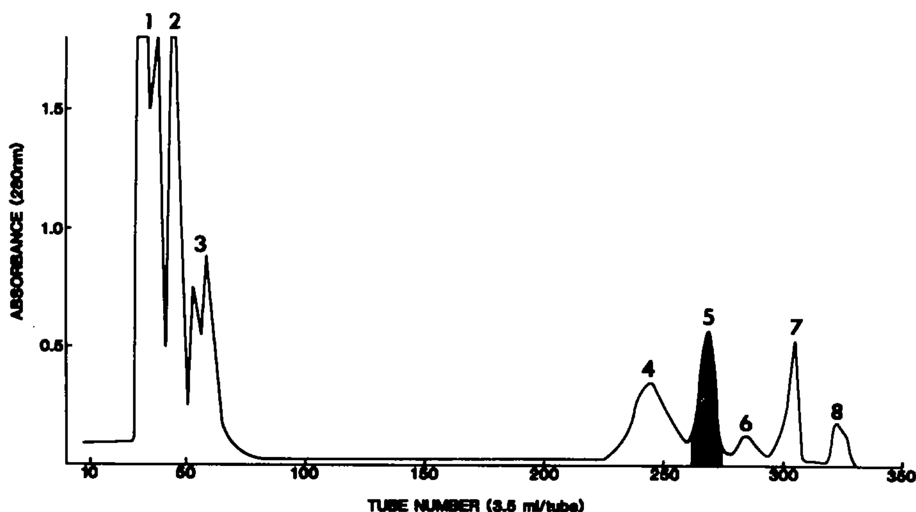

FIG. 1. ION EXCHANGE CHROMATOGRAPHY OF *B. asper* VENOM ON CM-SEPHADEX C-25. Venom (200 mg) was applied to the column which had been equilibrated with 0.05 M Tris, 0.1 M KCl (pH 7.0) buffer. The column was eluted with 200 ml of the same buffer. Then, a linear salt gradient of 0.05 – 0.75 M KCl was developed. Fractions of 3.5 ml were collected. Peak 5 (shaded) showed most of the myotoxic activity, and was further purified by gel filtration on Sephadex G-75.

polyacrylamide disc gel electrophoresis (Fig. 2). In some of the isolations, peak 5 of the CM-Sephadex chromatography had a minor impurity. In these cases, the toxin was purified by running a second CM-Sephadex column using the same conditions described.

Molecular weight estimation

Figure 3 shows the molecular weight estimation using gel filtration in Sephadex G-75. *B. asper* myotoxin has an estimated molecular weight of 10,700.

Immunodiffusion

The toxin formed a single precipitation band in the gel plates when tested against the polyvalent antivenom (Fig. 4). This band showed a complete immunological identity with one of the bands produced by the interaction of crude *B. asper* venom and antivenom. This precipitation band also showed identity with one band obtained between *B. atrox* crude venom and the antivenom. No immunological reaction was observed between *B. asper* myotoxin and anti-myotoxin *a* serum.

Myotoxic effect

Histologic evidence of myonecrosis, i.e. clumped myofibrillar material, was observed within 30 min after toxin injection (Fig. 5). At 30 min and 1 hr many cells showed a wedge-shaped lesion with the base located at the cell surface and the apex pointing to the interior of the fiber. These lesions are very similar to the "delta lesions" observed in other muscle pathologies (e.g. MOKRI and ENGEL, 1975). By 3 hr many cells were necrotic. By 6 hr the morphology of some fibers had become more hyaline with a homogenization of the myofibrillar material. At 6 and 12 hr phagocytic cells were present in the necrotic fibers while phagocytosis was well advanced by 24 and 48 hr (Fig. 5). There were no histological changes in nerves, muscle spindles, or blood vessels. On the other hand, the crude venom induced a typical picture of myonecrosis and hemorrhage with a strong inflammatory

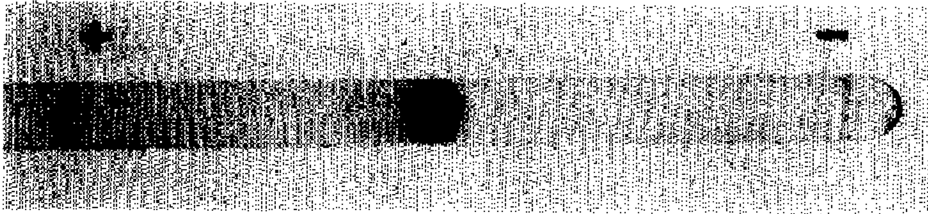

FIG. 2. POLYACRYLAMIDE GEL ELECTROPHORESIS OF *B. asper* MYOTOXIN.
Electrophoresis was carried out using 15% acrylamide at pH 4.3.

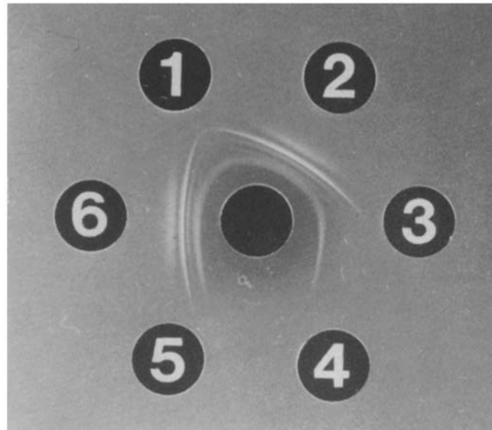

FIG. 4. OUCHTERLONY AGAROSE GEL-DIFFUSION PLATE.

Center well: polyvalent antivenom. Peripheral wells: (1) and (3) *B. asper* myotoxin (1 mg/ml); (2) *B. asper* venom (1 mg/ml); (4) myotoxin *a* (1 mg/ml); (5) physiologic saline solution; (6) *B. atrox* venom (1 mg/ml).

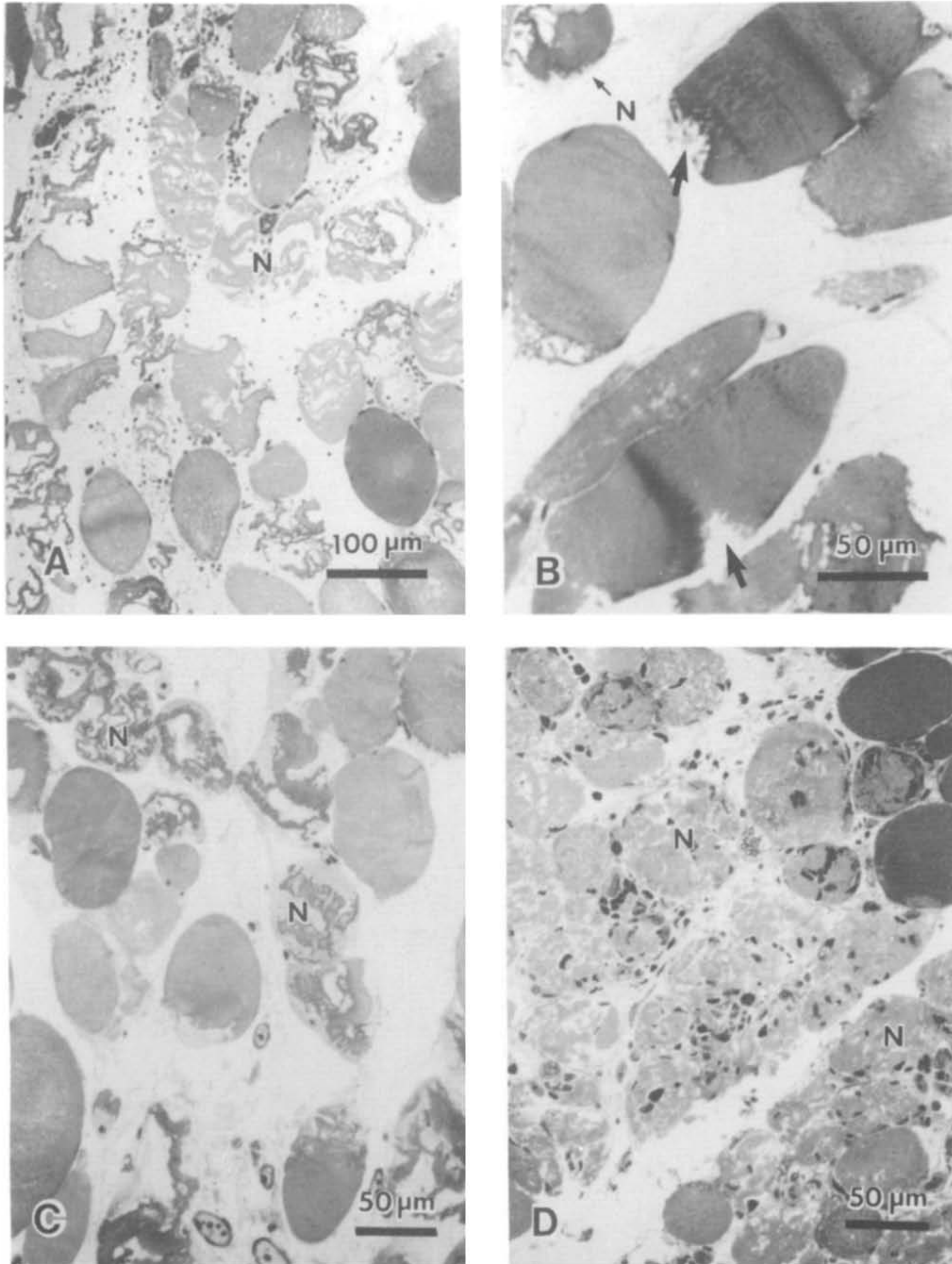

FIG. 5. PHOTOMICROGRAPHS OF MOUSE SKELETAL MUSCLE TAKEN AT VARIOUS TIME INTERVALS AFTER I.M. INJECTION OF *B. asper* VENOM (2.5 µg/g) OR TOXIN (2.5 µg/g).

(A) Venom, 1 hr. Note the presence of necrotic muscle cells (N), with clumped myofibrils. Hemorrhage is indicated by the presence of erythrocytes in the connective tissue. (B) Toxin, 30 min. Several cells show wedge-shaped lesions ("delta lesions") (arrow), whereas other cells are in a more advanced stage of degeneration (N). (C) Toxin, 3 hr. Necrotic muscle cells (N) show a conspicuous clumping of myofibrils. (D) Toxin, 24 hr. Most of the cells are necrotic (N), but the myofibrils are more homogeneous and hyaline in appearance. Phagocytic cells are observed within the necrotic fibers and in the interstitial space.

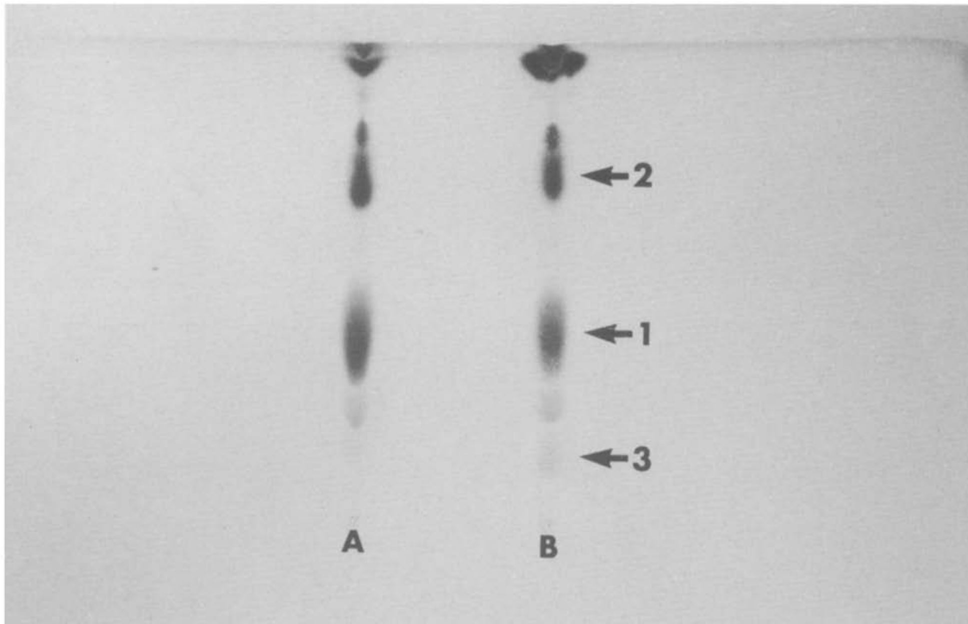

FIG. 8. THIN LAYER CHROMATOGRAM OF LIPID EXTRACTS FROM MUSCLE OF MICE INJECTED WITH PHYSIOLOGIC SALINE SOLUTION (A) AND MYOTOXIN AT A DOSE OF 5 μg/g (B).

(1) Phosphatidylcholine; (2) phosphatidylethanolamine; (3) lysophosphatidylcholine. The chromatographic separation was performed in precoated TLC silica gel 60F-254 plates (EM reagents). Plates were run in chloroform/methanol/acetic acid/water (54:25:8:4). They were then dried, sprayed with concentrated sulfuric acid – 30% formaldehyde (97:3 by volume) and heated at 90°C for 20 min.

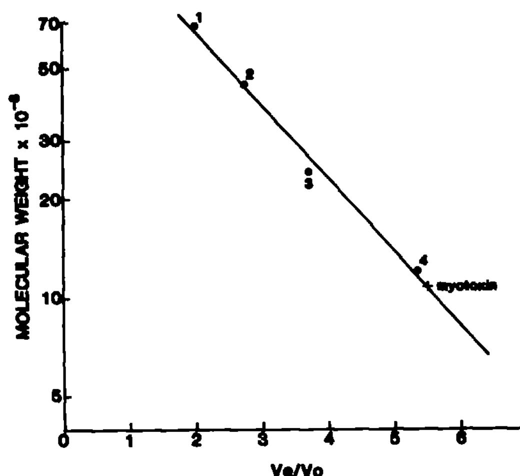

FIG. 3. MOLECULAR WEIGHT ESTIMATION OF *B. asper* MYOTOXIN BY GEL FILTRATION IN A SEPHADEX G-75 COLUMN (100 × 1.5 cm) USING 0.05 M TRIS, 0.1 M KCl (pH 7.0) AS ELUTION BUFFER. Standards: (1) bovine serum albumin, (2) ovalbumin, (3) chymotrypsinogen A, (4) cytochrome c. The estimated molecular weight of the myotoxin was 10,700.

reaction characterized by edema and the presence of phagocytic cells. A more detailed account of these histological findings will be presented elsewhere. The pattern of creatine kinase increase was very similar after crude venom and myotoxin injections. In both cases creatine kinase peaked at 3 hr and then decreased towards normal levels. However, the levels were higher at all times in mice injected with venom than in those injected with toxin (Fig. 6).

Calcium influx

The intracellular calcium levels increased markedly after toxin injection as judged by the increase in the differential absorbance of the Arsenazo III solution at 675 – 685 nm. There was a significant increase in absorbance in samples taken 30 min after injection, but the highest levels were observed in samples obtained 6 hr after inoculation (Fig. 7). The absorbance did not increase in solutions incubated with muscle obtained from control mice.

Inhibition by verapamil

Table 1 shows that verapamil was ineffective in neutralizing necrosis and the increase in plasma creatine kinase levels. Histologically, the sections obtained from mice injected with toxin alone were indistinguishable from the sections obtained from mice pretreated with verapamil and then injected with toxin.

Hydrolysis of phospholipids in vivo

Figure 8 shows that lysophosphatidylcholine was increased in muscle extracts from mice that had been injected with the toxin, as compared to muscle extracts from mice injected with saline solution. This lysophospholipid was detected in samples taken 30 min, 3 hr and 6 hr after inoculation.

Other activities of the toxin

B. asper myotoxin showed phospholipase A activity *in vitro*. When 2 μg of myotoxin

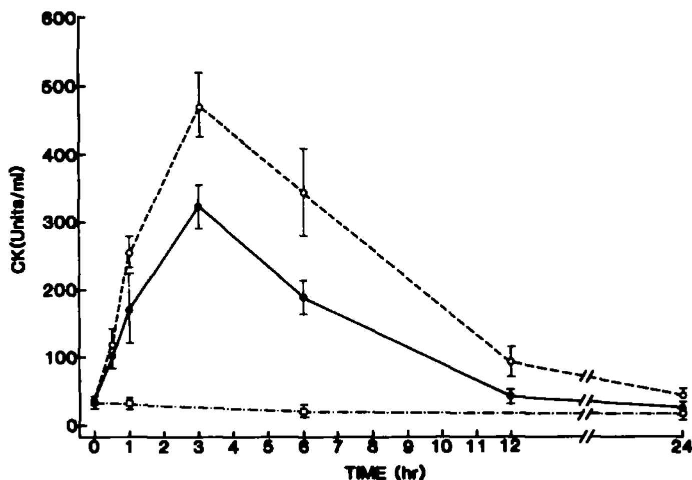

FIG. 6. PROFILE OF PLASMA CREATINE KINASE LEVELS IN MICE INJECTED I.M. WITH *B. asper* VENOM (2.5 μg/g) (O– – – –O), *B. asper* MYOTOXIN (2.5 μg/g) (●——●) AND PHYSIOLOGIC SALINE SOLUTION (□– – – –□).
One CK unit results in the phosphorylation of one nanomole of creatine per min at 25°C. Results are presented as mean ± S.E. (n = 4).

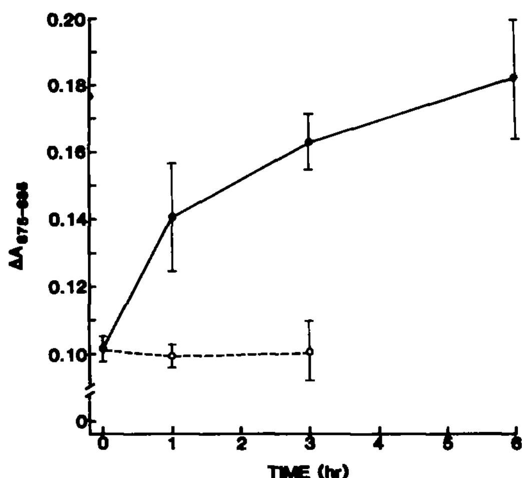

FIG. 7. INCREASE IN CALCIUM LEVELS IN MUSCLE AFTER I.M. INJECTIONS OF *B. asper* MYOTOXIN (5 μg/g). Muscle was obtained and blot dried. Approximately 50 mg of muscle were then transferred to a vial containing Arsenazo III 0.1 mM, digitonin 200 μg/ml, NaCl 140 mM, KCl 5 mM and imidazole 10 mM (pH 7.0) in a total volume of 2 ml. After exactly 1 min, 1 ml of the supernatant was transferred to a separate vial. The absorbance of the solution at 675 and 685 nm was read in a Beckman DU-8 spectrophotometer using a dual wavelength program. Calcium levels are directly proportional to the differential absorbance at 675 – 685 nm. (●——●) Mice injected with toxin; (O– –O) mice injected with physiologic saline solution. Results are presented as mean ± S.E. (n = 4).

(in 10 μl) were added to the wells of the egg yolk agarose plates, a clear halo of 18 mm diameter was observed after 20 hr of incubation at 50°C. When erythrocytes were added to the gel in order to increase specificity, the diameter of the hemolytic halo was the same as the diameter of the cleared egg yolk halo. The toxin induced indirect hemolysis in tubes.

TABLE 1. EFFECT OF VERAPAMIL ON MYOTOXICITY INDUCED BY *B. asper* TOXIN

Treatment*	Creatine kinase (units/ml)†	Myonecrotic index‡
PSS	28 ± 8	0
Verapamil (5 μg/g)	28 ± 3	0
Toxin (2.5 μg/g)	364 ± 36	0.38 ± 0.10
Pretreatment verapamil (10 μg/g); then toxin (2.5 μg/g)	384 ± 36	0.35 ± 0.06
Pretreatment verapamil (5 μg/g); then toxin (2.5 μg/g)	347 ± 37	0.42 ± 0.09
Pretreatment verapamil (2.5 μg/g); then toxin (2.5 μg/g)	387 ± 28	0.33 ± 0.12
Simultaneous injection verapamil (5 μg/g); toxin (2.5 μg/g)	345 ± 62	0.34 ± 0.12

*In pretreatment experiments verapamil was injected i.m. 5 min before the toxin. In simultaneous injections, verapamil and toxin were inoculated i.m. at the same time. PSS = physiologic saline solution.

†Expressed as units/ml ± S.E. (n = 4); one CK unit results in the phosphorylation of one nanomole of creatine per min at 25°C.

‡Myonecrotic index = number of necrotic cells/number of total cells. Expressed as mean ± S.E. (n = 8). Verapamil did not cause any significant change ($P > 0.1$) in the myonecrotic index when combined with the toxin.

When 10 μg of the toxin were added to the suspension of erythrocytes and lecithin, 87 ± 3% hemolysis (n = 4) was observed after 30 min of incubation. When the phospholipids were excluded from the mixture, no hemolysis was observed. Thus, the toxin is not directly hemolytic.

The myotoxin lacked proteolytic activity when tested by the highly sensitive hide powder azure substrate. Furthermore, the toxin was devoid of hemorrhagic activity, since both macroscopic and microscopic examination of muscular tissue revealed the absence of erythrocytes in the connective tissue space.

DISCUSSION

A homogeneous myotoxic phospholipase has been isolated from *B. asper* venom. The toxin induces myonecrosis in mice which resembles the action of the crude venom in two ways. First, the profile of creatine kinase levels in plasma is very similar, although the venom induces a greater elevation. Second, the histological picture observed after toxin injection partially resembles the histology of muscle inoculated with crude venom. However, the latter drastically affects the vasculature and induces a necrotic pattern that is not identical to the one observed after toxin injection. This is probably due to the action of other toxins (i.e. myotoxins and hemorrhagic toxins) present in the crude venom.

Several myotoxins have been isolated from snake venoms. They can be placed into three main groups: (a) The myotoxic phospholipases, such as notexin (HARRIS *et al.*, 1975), taipoxin (HARRIS and MALTIN, 1982), crotoxin (HAWGOOD, 1982), mojave toxin (GOPALAKRISHNAKONE *et al.*, 1980) and some isolated from Australian elapid venoms (MEBS and SAMEJIMA, 1980); (b) the "cardiotoxins", present in some elapid venoms (CHANG, 1979), which have a broad spectrum of pharmacological effects; (c) small basic toxins such as myotoxin *a* (OWNBY *et al.*, 1976), crotamine (CAMERON and TU, 1978),

a toxin isolated from *Crotalus adamanteus* (MEBS *et al.*, 1983) and toxins isolated from the venoms of *Crotalus durissus durissus* (Eneff, M. S. Thesis, Oklahoma State University, 1982) and *Crotalus viridis concolor* (POOL *et al.*, 1981). *Bothrops asper* myotoxin belongs to the first group, since it has phospholipase A activity and induces biochemical and histological alterations in skeletal muscle which resemble those induced by some myotoxic phospholipases, such as notexin (HARRIS *et al.*, 1975), taipoxin (HARRIS and MALTIN, 1982) and crotoxin (HAWGOOD, 1982). However, the relative potency of these myotoxins is very different, since toxins isolated from Australian elapid venoms (MEBS *et al.*, 1983) induce more drastic myonecrosis than crotoxin and *B. asper* myotoxin. Table 2 is a review of the characteristics of some myotoxins isolated from crotaline snake venoms.

TABLE 2. PROPERTIES OF SOME MYOTOXINS ISOLATED FROM CROTALINE SNAKE VENOMS

Toxin	Species	Molecular weight	Enzymatic activity	Other biological activities	Reference
Myotoxin *a*	*Crotalus viridis viridis*	4400		Hemolytic	CAMERON and TU (1977)
Crotamine	*Crotalus durissus terrificus*	4900			LAURE (1975)
Crotoxin	*Crotalus durissus terrificus*	24,000	Phospholipase A	Neurotoxic Hemolytic	BREITHAUPT *et al.* (1974)
Mojave toxin	*Crotalus scutulatus*	24,310	Phospholipase A	Neurotoxic	CATE and BIEBER (1978)
Viriditoxin	*Crotalus viridis viridis*	115,000	Proteolytic	Hemorrhagic	FABIANO and TU (1981)
B. asper myotoxin	*Bothrops asper*	10,700	Phospholipase A	Hemolytic	Present work

As regards the mode of action of the myotoxin, the following observations indicate that it may affect the integrity of the plasma membrane. (a) The toxin induces a rapid increase in plasma creatine kinase levels. (b) There is an elevation in cytosolic calcium levels after toxin injection. The experiments with verapamil indicate that this calcium influx does not occur through voltage-sensitive calcium channels which in skeletal muscle are located in the T tubules (ALMERS *et al.*, 1981). This influx is probably a consequence of a generalized plasma membrane disruption. (c) Preliminary ultrastructural studies show that there are focal disruptions in the integrity of the sarcolemma in tissue obtained 30 min after toxin injection. (d) Histologically, some cells show wedge-shaped lesions ("delta lesions") which have the base at the cell surface and the apex points towards the interior of the fibers. These lesions have been described in other muscle diseases (e.g. Duchenne muscular dystrophy, MOKRI and ENGEL, 1975). It has been shown that "delta lesions" represent focal areas of degeneration that underline portions of the cell where the plasma membrane is disrupted or lost. (e) There is an increase in the content of lysophosphatidylcholine in muscle after myotoxin injection. This might be due to the phospholipolytic activity of the toxin on muscle phospholipids. If this is the case, the toxin may be affecting the membrane integrity either by depleting the membrane phospholipids or by releasing lysophospholipids which are able to induce muscle necrosis themselves (PESTRONK *et al.*, 1982).

If the toxin acts by altering the structural and functional integrity of the plasma membrane, this would result in a generalized increase in membrane permeability to macromolecules and ions. The observed calcium influx is particularly significant since it has been repeatedly proposed that an increase in cytosolic calcium levels is a critical step in the process of cell death in a wide variety of pathological conditions (TRUMP *et al.*,

1981). In skeletal muscle, such an increase in calcium levels results in hypercontraction of the myofilaments, mitochondrial poisoning (WROGEMANN and PENA, 1976), activation of calcium-dependent proteases (DUNCAN, 1978) and phospholipases (TRUMP *et al.*, 1981) and eventually cell death.

Is the phospholipase A activity relevant to the myotoxic action of this toxin? Although this question cannot be answered with our data, it seems very likely that the phospholipolytic activity is related to myotoxicity. CONDREA *et al.* (1981) have questioned the correlation between phospholipase activity and toxicity in several phospholipases isolated from elapid venoms. Nevertheless, the fact that *B. asper* myotoxin is able to release lysolecithin *in vivo* and that this lipid can induce myonecrosis (PESTRONK *et al.*, 1982) supports the idea that this necrosis is at least partially due to the phospholipase activity of the toxin. HARRIS and MACDONELL (1981) demonstrated that notexin is able to hydrolyze phospholipids in rat skeletal muscle. They proposed that the myotoxicity of notexin is based on its phospholipase A activity. The same mechanism has been proposed for crotoxin (HAWGOOD, 1982) and taipoxin (HARRIS and MALTIN, 1982). Alternatively, the phospholipid hydrolysis observed *in vivo* could be the result of the activation of intracellular phospholipases. There are calcium-dependent phospholipases which play a role in membrane turnover (SHIER, 1982). Since *B. asper* myotoxin induces a calcium influx, a combination of extrinsic and intrinsic phospholipolytic activities may take place simultaneously in this pathologic event.

In conclusion, this myotoxin may prove helpful in obtaining a better understanding of muscle necrosis induced by *B. asper* venom and may contribute to the development of more specific and successful treatments.

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REFERENCES

ALMERS, W., FINK, R. and PALADE, P. T. (1981) Calcium depletion in frog muscle tubules: the decline of calcium current under maintained depolarization. *J. Physiol., Lond.* **312**, 177.

ARROYO, O. and GUTIÉRREZ, J. M. (1981) Ultraestructura del efecto mionecrótico inducido por el veneno de *Bothrops asper* de Costa Rica en ratón blanco. *Toxicon* **19**, 773.

BOLAÑOS, R. and CERDAS, L. (1978) The production and control of anti-venoms sera. In: 15th IABS Congress: vaccinations in the developing countries. La Guadeloupe. *Devs biol. Standard.*, Vol. 41, pp. 109–114.

BREITHAUPT, H., RUBSAMEN, K. and HABERMANN, E. (1974) Biochemistry and pharmacology of the crotoxin complex. Biochemical analysis of crotapotin and the basic crotalus phospholipase A. *Eur. J. Biochem.* **49**, 333.

CAMERON, D. and TU, A. T. (1977) Characterization of myotoxin *a* from the venom of prairie rattlesnake (*Crotalus viridis viridis*). *Biochemistry* **16**, 2546.

CAMERON, D. and TU, A. T. (1978) Chemical and functional homology of myotoxin *a* from prairie rattlesnake venom and crotamine from South American rattlesnake venom. *Biochim. biophys. Acta* **532**, 147.

CATE, R. L. and BIEBER, A. L. (1978) Purification and characterization of Mojave (*Crotalus scutulatus scutulatus*) toxin and its subunits. *Archs Biochem. Biophys.* **189**, 397.

CHANG, C. C. (1979) The action of snake venoms on nerve and muscle. In: *Snake Venoms*, pp. 309–376 (LEE, C. Y., Ed.). Berlin: Springer.

CLAUSEN, J. (1969) Immunochemical techniques for the identification and estimation of macromolecules. In: *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 2, Part III (WORK, T. S. and WORK, E., Eds). Amsterdam: North Holland.

CONDREA, E., FLETCHER, J. E., RAPUANO, B. E., YANG, C. C. and ROSENBERG, P. (1981) Dissociation of enzymatic activity from lethality and pharmacological properties by carbamylation of lysines in *Naja nigricollis*

and *Naja naja atra* snake venoms phospholipases A_2. *Toxicon* **19**, 705.

DUNCAN, C. J. (1978) Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. *Experientia* **34**, 1531.

FABIANO, R. and TU, A. T. (1981) Purification and biochemical study of viriditoxin, tissue damaging toxin, from prairie rattlesnake venom. *Biochemistry* **20**, 21.

GOPALAKRISHNAKONE, P., HAWGOOD, B. J., HOLBROOKE, S. E., MARSH, N. A., SANTANA DE SA, S. and TU, A. T. (1980) Sites of action of mojave toxin isolated from the venom of the Mojave rattlesnake. *Br. J. Pharmac.* **69**, 421.

GUTIÉRREZ, J. M., ARROYO, O. and BOLAÑOS, R. (1980) Mionecrosis, hemorragia y edema inducidos por el veneno de *Bothrops asper* en ratón blanco. *Toxicon* **18**, 603.

GUTIÉRREZ, J. M., CHAVES, F., BOLAÑOS, R., CERDAS, L., ROJAS, E., ARROYO, O. and PORTILLA, E. (1981) Neutralización de los efectos locales del veneno de *Bothrops asper* por un antiveneno polivalente. *Toxicon* **19**, 493.

HABERMANN, E. and HARDT, K. L. (1972) A sensitive and specific plate test for the quantitation of phospholipases. *Analyt. Biochem.* **50**, 163.

HARRIS, J. B. and MACDONELL, C. A. (1981) Phospholipase A_2 activity of notexin and its role in muscle damage. *Toxicon* **19**, 419.

HARRIS, J. B. and MALTIN, C. A. (1982) Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus*. *Br. J. Pharmac.* **76**, 61.

HARRIS, J. B., JOHNSON, M. A. and KARLSSON, E. (1975) Pathological responses of rat skeletal muscle to a single subcutaneous injection of a toxin isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. *Clin. exp. Pharmac. Physiol.* **2**, 383.

HAWGOOD, B. J. (1982) Physiological and pharmacological effects of rattlesnake venoms. In: *Rattlesnake venoms. Their actions and treatment* (TU, A. T., Ed.). New York: Marcel Dekker.

JENG, T. W., HENDON, R. A. and FRAENKEL-CONRAT, H. (1978) Search for relationships among the hemolytic, phospholipolytic, and neurotoxin activities of snake venoms. *Proc. natn. Acad. Sci. U.S.A.* **75**, 600.

JIMÉNEZ, E. and GARCÍA, I. (1969) Análisis de 86 casos de ofidismo en niños. *Revta méd. Hosp. nac. Niños* **4**, 91.

LAURE, C. J. (1975) Die primarstruktur des Crotamins. *Hoppe-Seyler's Z. physiol. Chem.* **356**, 213.

MEBS, D. and SAMEJIMA, Y. (1980) Purification, from Australian elapid venoms, and properties of phospholipases A which cause myoglobinuria in mice. *Toxicon* **18**, 443.

MEBS, D., EHRENFELD, M. and SAMEJIMA, Y. (1983) Local necrotizing effect of snake venoms on skin and muscle: relationship to serum creatine kinase. *Toxicon* **21**, 393.

MOKRI, B. and ENGEL, A. G. (1975) Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. *Neurology* **25**, 1111.

MURPHY, E., COLL, K., RICH, T. L. and WILLIAMSON, J. R. (1980) Hormonal effects on calcium homeostasis in isolated hepatocytes. *J. biol. Chem.* **255**, 6600.

OWNBY, C. L., CAMERON, D. and TU, A. T. (1976) Isolation of myotoxic component from rattlesnake (*Crotalus viridis viridis*) venom. Electron microscopic analysis of muscle damage. *Am. J. Path.* **85**, 149.

OWNBY, C. L., WOODS, W. M. and ODELL, G. V. (1979) Antiserum to myotoxin from prairie rattlesnake (*Crotalus viridis viridis*) venom. *Toxicon* **17**, 373.

OWNBY, C. L., ODELL, G. V., WOODS, W. M. and COLBERG, T. R. (1983) Ability of antiserum to myotoxin *a* from prairie rattlesnake (*Crotalus viridis viridis*) venom to neutralize local myotoxicity and lethal effects of myotoxin *a* and homologous crude venom. *Toxicon* **21**, 35.

PESTRONK, A., PARHAD, I. M., DRACHMAN, D. B. and PRICE, D. L. (1982) Membrane myopathy: morphological similarities to Duchenne muscular dystrophy. *Muscle Nerve* **5**, 209.

PICADO, C. (1931) *Serpientes venenosas de Costa Rica. Sus venenos. Seroterapia antiofídica.* San José. Imprenta Alsina.

POOL, W. R., ENGLE, C. M. and BIEBER, A. L. (1981) Isolation and characterization of two myotoxins from the venom of the midget faded rattlesnake (*Crotalus viridis concolor*). *Fedn Proc. Fedn Am. Socs exp. Biol.* **40**, 1579.

REISFELD, R. A., LEWIS, U. J. and WILLIAMS, D. E. (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature, Lond.* **195**, 281.

RINDERKNECHT, H., GEOKAS, M. C., SILVERMAN, P. and HAUERBACK, B. J. (1968) A new ultrasensitive method for the determination of proteolytic activity. *Clin. chim. Acta* **21**, 197.

SCARPA, A., BRINLEY, F. J., TIFFERT, T. and DUBYAK, G. R. (1978) Metallochromic indicators of ionized calcium. *Ann. N.Y. Acad. Sci.* **307**, 86.

SHIER, W. T. (1982) Cytolytic mechanisms: self-destruction of mammalian cells by activation of endogenous hydrolytic enzymes. *J. Toxic.—Toxin Rev.* **1**, 1.

TRUMP, B. F., BEREZESKY, I. K. and OSORNIO-VARGAS, A. R. (1981) Cell death and the disease process. The role of calcium. In: *Cell Death in Biology and Pathology* (BOWEN, I. D. and LOCKSHIN, R. A., Eds.). London: Chapman and Hall.

TU, A. T. and HOMMA, M. (1970) Toxicologic study of snake venoms from Costa Rica. *Toxic. appl. Pharmac.* **160**, 73.

WROGEMANN, K. and PENA, S. D. J. (1976) Mitochondrial calcium overload: a general mechanism for cell necrosis in muscle diseases. *Lancet* **I**, 672.