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FASCÍCULO EM HOMENAGEM AO 70º ANIVERSÁRIO DO DR. SAUL SCHENBERG, DIRETOR DO SERVIÇO DE FISIOLOGIA DO INSTITUTO BUTANTAN.

THIS NUMBER IS A TRIBUTE TO DR. SAUL SCHENBERG, DIRECTOR OF THE DEPARTMENT OF PHYSIOLOGY, INSTITUTO BUTANTAN, ON OCCASION OF HIS 70th BIRTHDAY.

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STUDIES ON THE CROSS-REACTIVITY OF SNAKE VENOM ANTISERA

D. MEBS*
F. KORNALIK**

ABSTRACT: Cross-reactivity between antisera and snake venoms which have not been used for immunization is a common phenomenon observed in closely related as well as in quite distinct snake species. A polyvalent antivenom (Behringwerke North-Africa) was found to possess considerable neutralization potency to a North-American crotalid snake *(Crotalus adamanteus)*. By fractionation of *Bitis arietans* and of *Crotalus adamanteus* venom hemorrhagic factors were isolated which were neutralized by the antivenom indicating that there exist commom antigenic properties in venom constituents of even unrelated snake species.
KEY WORDS: Snake venom, antivenom cross-reactivity, hemorrhagic factors.

INTRODUCTION

Snake venoms are complex mixtures of proteins and polypeptides, which have antigenic properties. Immunesera raised against snake venoms contain, therefore, a great number of antibodies, of which some are important to neutralize the venom's lethal effect. Cross-reactivity of antivenoms with venoms of snakes which have not been used in immunization is a common phenomenon[1]. This leads to the assumption that venoms of even unrelated snakes representing different species or genera, or which are geographically separated may have common antigens.

The immunologic analysis of such complex mixtures of antigens and antibodies can be performed by applying immunodiffusion techniques. Precipitation lines, the reaction product between antigen and antibody, permit conclusions about the extent of relationship between venoms. A number of studies exist providing informations on interrelationships between species, on evolutionary and genetic implications[1]. However, the data obtained

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by these methods have limited value, if practical aspects of cross-reactivity, i.e. cross-neutralization, are concerned. The neutralization of the lethal and other harmful effects of snake venoms by antivenoms, which are paraspecific to others not used in immunization can only be evaluated by in vivo test-systems. This includes the neutralization of lethality, LD_{50}, hemorrhagic, necrotizing, defibrinating or coagulant activities in mice or rats. Whereas data on the neutralization capacity of commercial antivenoms to the lethal action of various snake venoms exist, neutralization studies on other venom activities are still rare. The WHO standardization program should certainly contribute to the knowledge on the potential cross-neutralization capacity of antivenoms.[2]

Beside these basic informations: which antivenom reacts with which venom, it appears important to find an answer to the questions: what characterizes common antigens and what is their role in the course of envenomation. It is quite clear that the main approach to this problem should concentrate on those venom components (antigens) only, which are involved in the venom's lethal or pathological actions.

In studies on various commercial antivenoms, their neutralizing capacity to venom activities such as lethal, hemorrhagic, necrotizing, defibrinating and coagulant effects, striking cross-reactivities had been observed[3,4,5] For instance Behringwerke-antivenom North-Africa as well as Wyeth Anticrotalidae-antivenom were both found to be very effective in neutralizing the lethal activity of the venom from the African puff adder (*Bitis arietans*). But it has to be considered that the polyvalent Behringwerke-antivenom only was produced by using *Bitis gabonica* venom as antigen among other venoms such as from *Naja haje, Vipera lebetina* and *Echis carinatus*, whereas in Wyeth-antivenom only North-and Central-American crotalid snake venoms, such as from *Crotalus atrox, C.adamanteus, C.durissus terrificus* and *Bothrops atrox* had been used for immunization. On the other hand, Wyeth-antivenom was much less effective to *Crotalus adamanteus* venom, but Behringwerke-antivenom shows a four times higher activity in the mouse protection test. Therefore, one may conclude that a common antigen (or antigens) responsible for the lethal effect must be present in both venoms.

Bitis arietans as well as *Crotalus adamanteus* venom produce massive hemorrhage which eventually leads to death (at least in mice), if internal bleeding occurs. Consequently the efficacy of both antivenoms to neutralize hemorrhagic activity was tested.

MATERIAL AND METHODS

Bitis arietans venom was purchased from D. Muller, Johannesburg, South Africa; *Crotalus adamanteus* venom was obtained from F. Kornalik. The following antivenoms were used for neutralization studies: polyvalent Crotalidae antivenom from Wyeth Laboratories, Philadelphia, PA, USA; polyvalent antivenom North-Africa from Behringwerke, Marburg, FRG. LD_{50} was assayed by intravenous injection into mice (20 g, 10 animals per dose), neutralization capacity of antivenoms by preincubating 0.1 ml venom solution (0.9% NaC1) with 0.1 ml antiserum for 30 min at 37° C. Hemorrhagic ac-

tivity was tested in mice according to Taborska[6]. Prior intradermal venom application 0.2 ml of 51_{Cr} labled mice erythrocytes were injected intravenously. In the neutralization tests 50 µg venom in 0.1 ml saline were mixed with 0.1 ml antivenom or normal rabbit serum as control and, after 30 min incubation, injected intradermally in the interscapular region of mice. The animals were sacrificed after 24 hrs and the amount of extravasal blood of a 2.5 cm^3 area at the injection site was calculated from radioactivity. Venom fractionation was performed as described previously[7].

RESULTS AND DISCUSSION

Under the conditions described Behringwerke-antivenom is able to completely neutralize the hemorrhagic activity of *Bitis arietans* and almost completely that of *Crotalus adamanteus* venom (Fig. 1). Wyeth-antivenom is less active towards *Bitis* venom, but provides full protection in the case of *Crotalus adamanteus* venom.

To identify the antigen or antigens involved in this cross-reaction *Crotalus adamanteus* venom was fractionated by gel filtration (Fig. 2). Three main protein-containing fractions were separated. Fraction I contained the hemorrhagic principle of the venom, fraction II several enzymes (proteases, phospholipase A) and fraction III represents a toxin of crotamine-like activity which is rarely found in *Crotalus adamanteus*, but is present in other *Crotalus* venoms[8]. It has myotoxic activity producing local myonecrosis like myotoxin a from *C. viridis viridis* venom. The last two fractions are protein-free and consist of nucleotides or similar compounds. When the LD_{50} of the fractions was determined by i.v. injection, only fraction I and III exhibited marked lethality, whereas fraction II was essentially non-toxic. Since similar toxins like the myotoxin of fraction III are not present in *Bitis* venom, it was assumed that the hemorrhagic factors in both venoms may be responsible for causing cross-reactions. Preliminary experiments demonstrated that fraction III is not neutralized by both antivenoms.

Since fraction II is essentially non-toxic, it may play a minor role in envenomation. Therefore, the hemorrhagic principle present in fraction I was further purified by ion-exchange chromatography on DEAE-cellulose, the hemorrhagin obtained was a semi-pure product which was compared to a hemorrhagin previously isolated from *Bitis arietans* venom[7]. Immunodiffusion tests using the Ouchterlony method revealed that both crude venoms, *Bitis arietans* and *Crotalus adamanteus*, produce several precipitation lines when reacting with Behringwerke-antivenom (Fig. 3). But also the semi-pure hemorrhagins from both venoms show distinct bands. Moreover, the non-toxic fraction II produced much more precipitation lines indicating that criteria for cross-reactivity based only on immunodiffusion tests are less useful.

In preliminary tests using 50 µg of each hemorrhagin mixed with 0.1 ml antivenom Behringwerke-antivenom was more effective (1:8 dilution) in neutralizing *Crotalus adamanteus* than *Bitis arietans* hemorrhagin (1:4 dilution). Wyeth-antivenom exhibited full protection (more than 1:8 dilution) to *Crotalus adamanteus*, but was ineffective to *Bitis arietans* hemorrhagin.

What are the conclusions from these experiments? Snake venoms con-

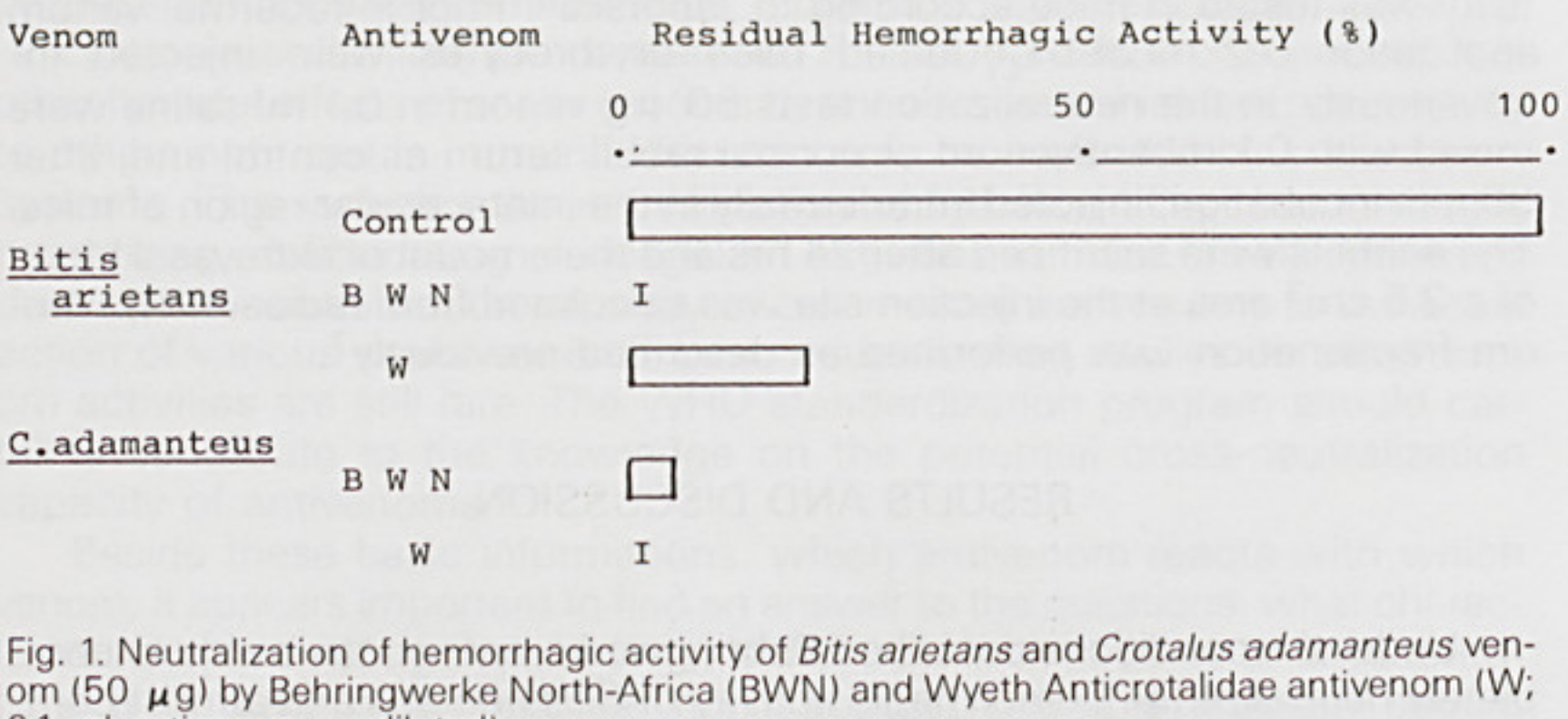

Fig. 1. Neutralization of hemorrhagic activity of *Bitis arietans* and *Crotalus adamanteus* venom (50 μg) by Behringwerke North-Africa (BWN) and Wyeth Anticrotalidae antivenom (W; 0.1 ml antivenom, undiluted).

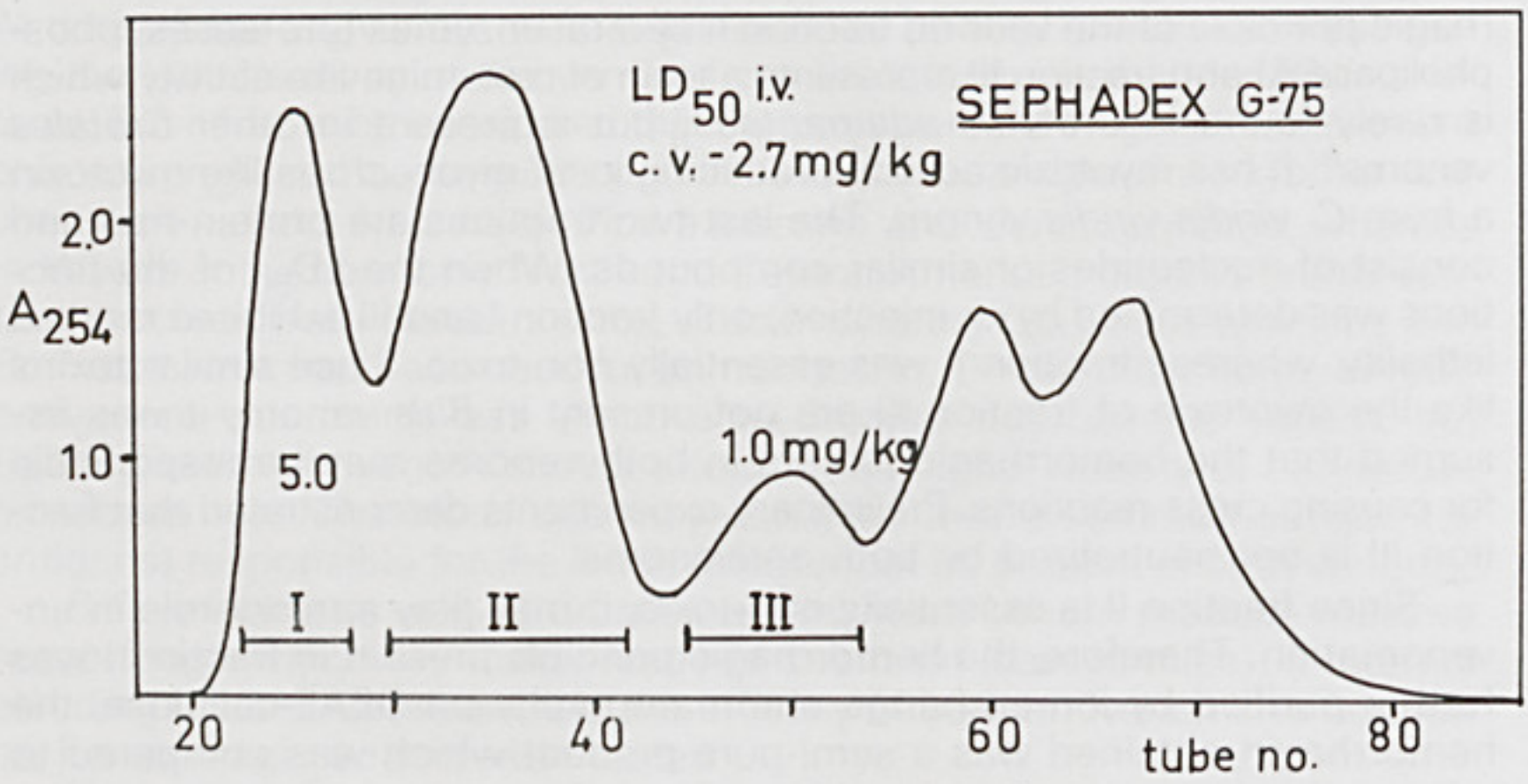

Fig. 2. Gel filtration of 500 mg *Crotalus adamanteus* venom on a Sephadex G-75 column (95 x 2.5cm) eluted with 0.1M ammonium acetate. Fractions of 6 ml were collected at a flow rate of 36ml per hr. LD_{50} values of crude venom (c.v.) and fraction I and III are shown in the elution pattern, fraction II is essentially non-toxic.

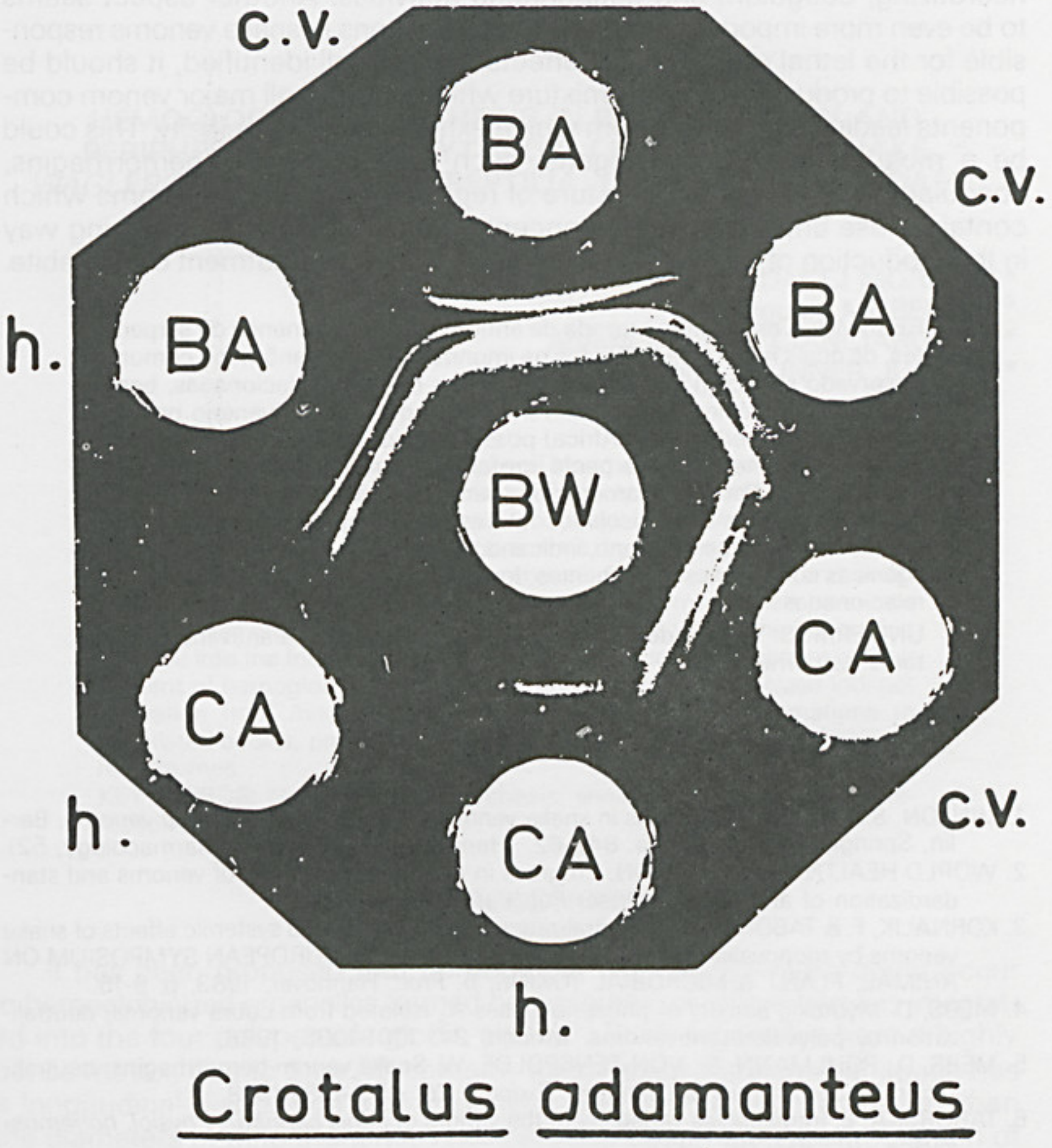

Fig.3. Immunodiffusion-test of *Bitis arietans* (BA), *Crotalus adamanteus* (CA) and of their semi-pure hemorrhagins (h.) to Behringwerke North-Africa (BW) antivenom.

tain common antigens which are of minor importance in envenomation, but others which may also be involved in the venom's lethal or noxious action. From a practical point of view, demonstration of cross-neutralization seems to be of great value, if a wider application of polyspecific antivenoms in snakebite cases is concerned. On the other hand, our present knowledge on cross-reactivity and neutralization is rather poor, if one considers the number of commercial antivenoms available and the tests which could be done with snake venoms involved in human envenomations. Beside lethality-neutralization this should include other test systems such as hemorrhagic, necrotizing, coagulant and defibrinating activities. Another aspect seems to be even more important. Since common antigens in snake venoms responsible for the lethal or pathologic effects can be well identified, it should be possible to produce an antigen mixture which includes all major venom components leading to an antivenom which exhibits broad specificity. This could be a mixture of purified antigens, such as neurotoxins, hemorrhagins, coagulant factors etc., or a mixture of representative snake venoms which contain these antigens in high concentrations. It may be a promising way in the production of more effective antivenoms for the treatment of snakebite.

RESUMO: A reatividade cruzada de antivenenos com venenos de serpentes, os quais não foram utilizados na imunização, é um fenômeno comum observado seja com espécies de serpentes bastante relacionadas, bem como com espécies diferentes. Verificou-se que um antiveneno polivalente (Behringwerke North-Africa) possui considerável potência neutralizante para veneno de serpente crotalidea norte-americana *(Crotalus adamanteus)*. Por fracionamento dos venenos de *Bitis arietans* e de *Crotalus adamanteus* foram isolados fatores hemorrágicos, os quais foram neutralizados pelo antiveneno, indicando a existência de propriedades antigênicas comuns nos constituintes dos venenos, mesmo de espécies não relacionadas.

UNITERMOS: Veneno de serpente, reatividade cruzada de antiveneno, fatores hemorrágicos.

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HEMOGLOBIN BIOSYNTHESIS IN BONE MARROW AND PERIPHERAL BLOOD ERYTHROID CELLS OF THE SNAKE *WAGLEROPHIS MERREMII* (REPTILIA, OPHIDIA, COLUBRIDAE)*

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Pasquale MORENA***
Aurora Marques CIANCIARULLO**
Adolpho BRUNNER JUNIOR**

ABSTRACT: Bone marrow and peripheral blood erythroid cells of the snake *Waglerophis merremii* were ultrastrùcturally analysed. To enhance the entry of immature cells to the peripheral blood, erythropoiesis was stimulated through the induction of a hemolytic anemia, by injecting saponin. Typical hemosomes, very similar to those observed in mammalian and avian immature erythroid cells were found, as well as some of their precursor forms. These organelles are taken as possible sites where the integration of heme into the four globin polypeptide chains occur, on account of their content of hemoglobinic nature. Such entities are a specialyzed indirect derivation from mitochondria, through successive transformations to lamellated bodies, prehemosomal vesicles, prohemosomes and finally to hemosomes.

KEY WORDS: Hemoglobin biosynthesis; snake hemoglobin; snake hemosome.

INTRODUCTION

It has been proposed that hemoglobin (*Hb*) biosynthesis could occur in hemoglobinized organelles termed hemosomes, or where heme is integrated into the four polypeptide globin chains[1]. Such organelles have a highly dense matrix, due to their *Hb* content, their narrow intralamellar space has a longitudinal disposition and in general their diameters are smaller than the diameters of mitochondria. These studies began by the examination of

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rabbit-embryo peripheral blood erythroblasts and reticulocytes. Further investigations were carried out in order to ascertain the hypothesis on the functional role of hemosomes. Several experimental and analytical works showed circumstantially a constant close relationship between *Hb* biosynthesis and hemosome formation[2-5,7,8]. Hemosomes were also found in mouse[11,12,14], other rodents[13], chicken[15], toads (*Bufo ictericus* and *Bufo paracnemis*)[10], as well as in the fish *Thymallus thymallus* erythroid cells[14]. It has been pointed out that this characteristic organelle is an indirect derivation of the mitochondrion[7,9], that occurs through successive modifications to lamellated body, prehemosomal vesicle, prohemosome, and hemosome.

This paper shows that *Waglerophis merremii* erythroid cells contain hemoglobinized organelles, through cell fractionation and electrophoresis of the organellar lysate supernatant. Results of electron microscopic studies on some stages of hemosome formation are presented, as already partially shown[19], comparatively to the findings in erythroid cells of other vertebrates.

MATERIAL AND METHODS

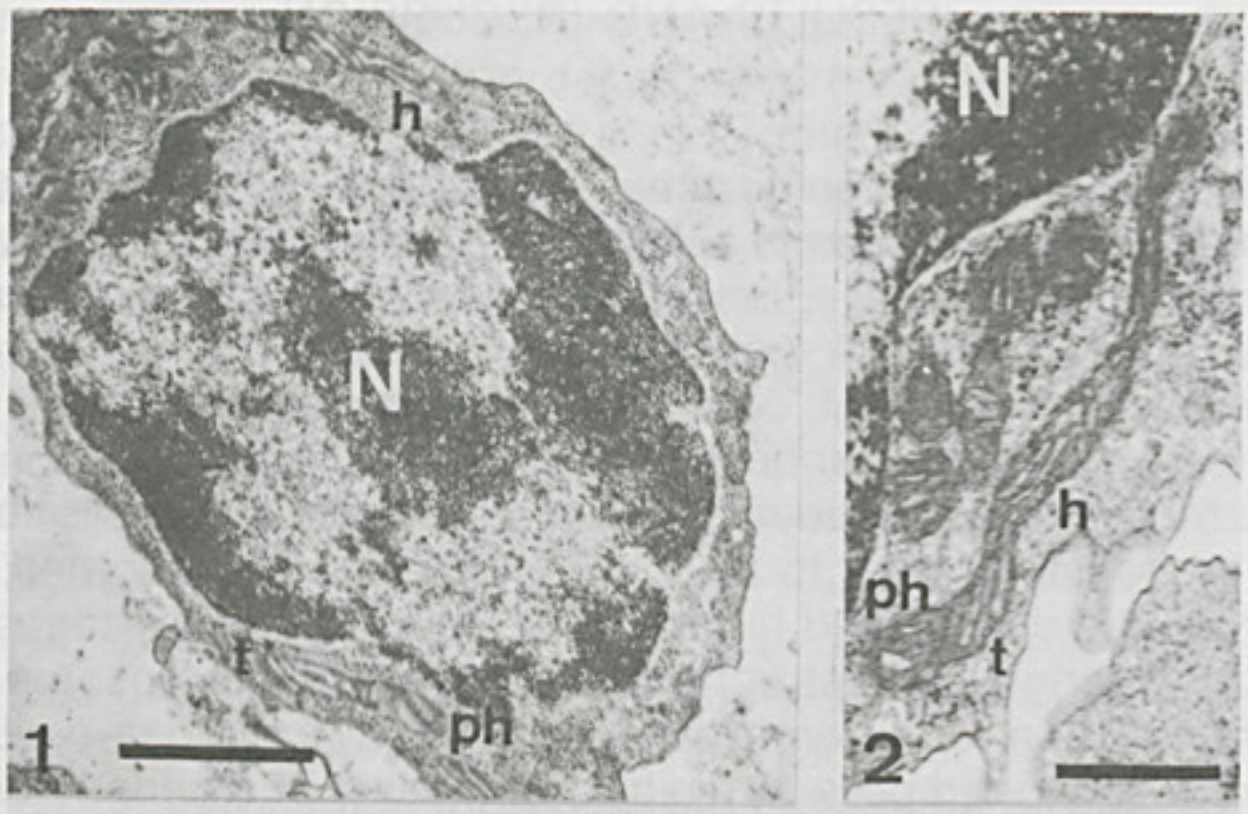
Normal adult snakes (*Waglerophis merremii*) newly received by the Instituto Butantan were submitted to hemolytic anemia. The snakes were subcutaneously injected, each day with saponin in 0.75% saline solution, 3 mg/kg body weight, for three days; blood was harvested (72 h), and reticulocyte countings reached approximately 30%, as examined through a modified new methylen blue technique[18]. For electron microscopy, blood was fixed initially in 1% glutaraldehyde in 0.20 M Millonig's buffer, for 1 h at room temperature, in order to concentrate the immature forms in the course of normal sedimentation. After this, erythroid cells were fixed in 3% glutaraldehyde, for approximately 2 h at room temperature, followed by osmic tetroxide fixation, 1% in the same buffer for 1 h at 4°C, uranyl acetate contrastation, 40 min at room temperature, dehydration, and embedding in Polylite 8001[9]. Thin sections were obtained in a Porter-Blum MT-1 microtome, contrasted by lead citrate and examined in the Elmiskop I and Zeiss-EM 109 electron microscopes, at 60 and 80 kV, from × 7,000 to × 50,000 magnification.

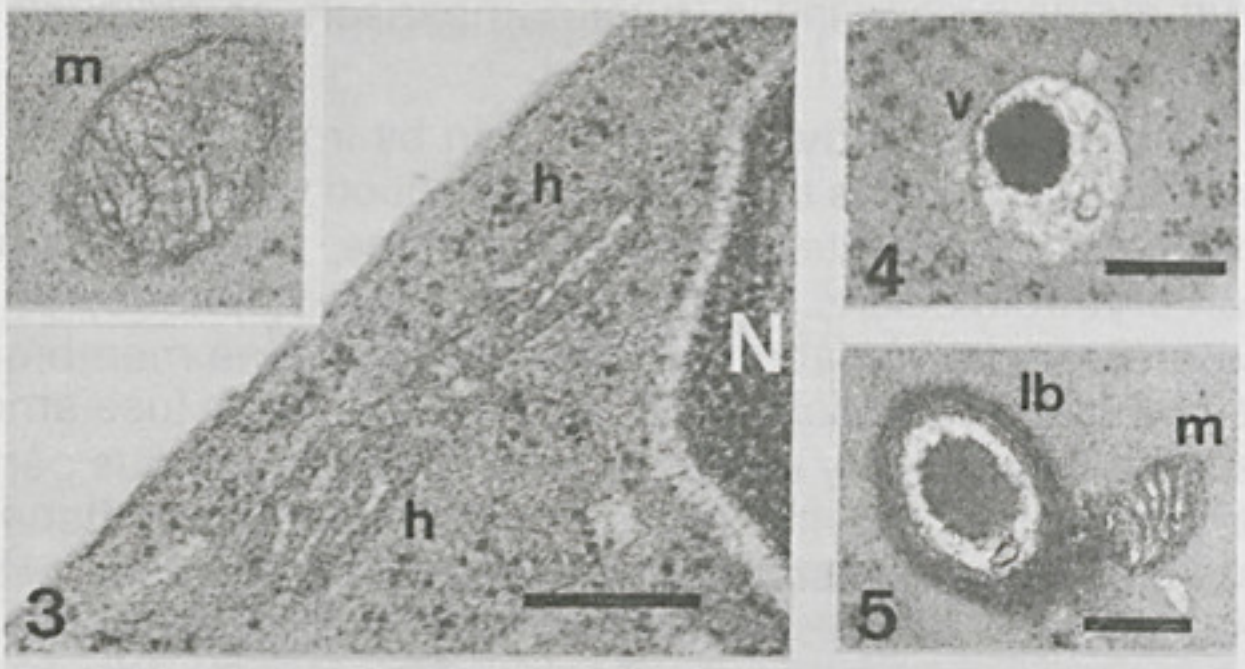
For the demonstration of *Hb* within the hemosomes through electrophoresis in polyacrylamide gel[16], blood cells were fractionated, the hemosomes isolated, washed and osmotically lysed, following Brunner *et al.*[1] with some modifications: Centrifugation at 4,000 xg, instead of 1,350 xg, for a complete nucleus and cell debris sedimentation without repetition of this step; centrifugation at 12,000 xg, instead of 10,000 xg, for the organellar fraction sedimentation. The supernatants of the organellar lysate and of the last washing were concentrated ten fold, or lyophilized, and *Hb* was diluted to 1:50. A 2.5mA current was applied for 40 min at 5°C, and *Hb* bands were identified by benzidine or tolidine reaction.

RESULTS AND DISCUSSION

The interpretation given for the several structural aspects found in *Waglerophis merremii* erythroid cells synthesizing *Hb* and involved in hemosome formation, is basically fundamented on studies carried out in mammalian erythroid cells[9]. A general view of a maturing bone marrow erythroblast is shown in Fig.1, corresponding to a still highly immature stage, as evaluated by the high number of polysomes and single ribosomes. Some organelles modifying continuously from prohemosomes to transitional stages followed by transformation to the hemoglobinized hemosomes, are seen. A long organelle constituted by the three developmental stages is shown in Fig.2, presenting a great structural similarity to the hemosomes and their precursors found in rabbit-embryo[1], adult rabbit[6,8], guinea-pig[3], human[2], and chicken[15] erythroid cells. A typical hemosome of a peripheral blood immature erythrocyte is seen in Fig.3; for comparison, an obliquely sectioned mitochondrion is presented in the inset.

As to *Hb* biosynthesis dynamics it begin by iron uptake via transferrin which constitute complexes through an interaction with glycoprotein receptors bound at numerous sites of the cell surface, probably as happens in mammalian erythroid cells[17]. This occurs through rhopheocytosis, consisting in a progressive invagination process of the plasma membrane (Fig.8), giving rise to endocytic vesicles (Fig. 6). These vesicles fuse among themselves resulting large, highly electron-dense free ferruginous particle inclusions, due to the disappearance of the limiting membrane. Afterwards, such ferruginous inclusions, constituting an iron source for heme biosynthesis, are involved by an initially single membrane (Fig.4); the vesicle content becomes amorphous and less electron-dense. The membrane which involves the ferruginous material results from the unfolding of double lamellae originated from a lamellated body that in turn is a mitochondrial derived structure (Fig.5), as shown in tissue culture cells[7] and in rabbit erythroblasts[9]. This lamellated body is a structural as well as probably an enzymatic complex catalysing heme and the final *Hb* biosynthesis reactions. From this stage onwards the captation of iron containing compounds, and of the globin chains occur through double lamellae expansions. These expansions return constituting prehemosomal vesicles which, possibly after a rotational movement, approaches the ferruginous material and the globin chains one another, giving rise to condensed prehemosomal vesicles (Fig. 6) that afterwards modify successively to prohemosomes (Fig. 7) and hemosomes[9]. At this stage the integration of heme into the four globin chains could take place. When hemosomes attain a high *Hb* concentration, their membranes disrupt and *Hb* spread throughout the cytoplasm. This happens repeatedly until the final cytoplasmic *Hb* concentration is reached for the constitution of a new mature erythrocyte. Claussen *et al.*[13] and Claussen[14] studying erythropoiesis in the rodents *Apodemus sylvaticus* and *Micromys minutus,* in the mouse *Mus musculus* and in the fish *Thymallus thymallus* found very interesting ultrastructural aspects of *Hb* biosynthesis similar to those found in the course of red blood cell maturation in other vertebrates. However, there exists a little disagreement between Claussen's[14] diagram of *Hb* biosynthesis and the interpretation of Brunner *et al.*[9] as to the integration of the globin chains





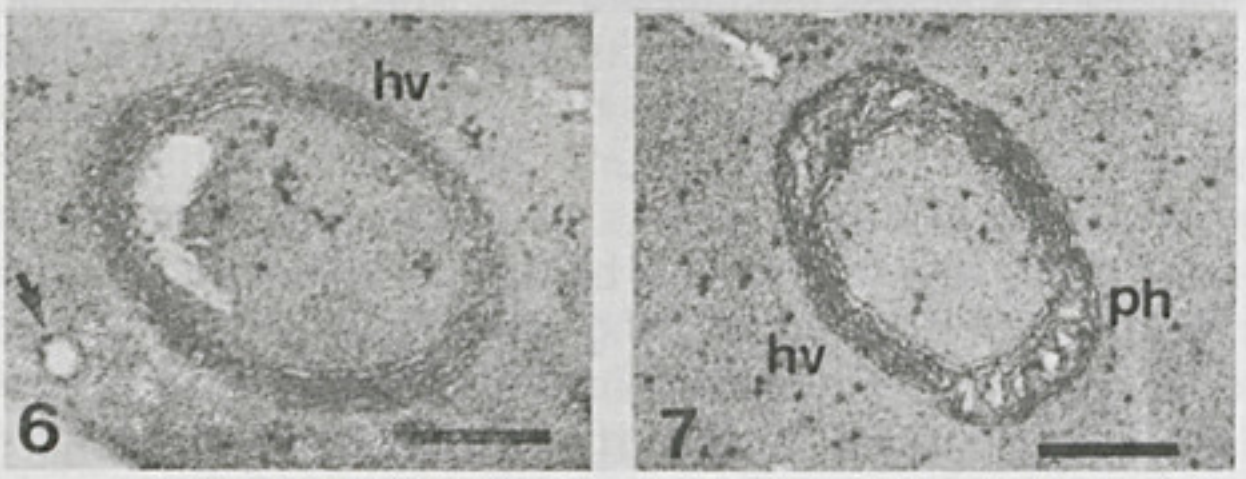


Fig. 1 — Bone marrow erythroblast showing a prohemosome *ph* continuous to a transitional stage *t*. At the upper region of the picture, the continuity of this stage *(t)* with the hemosome *h* can be seen; *N* — nucleus. Bar equals 1.0 μm.

Fig. 2 — Larger magnification of an erythroblast showing a long organelle constituted by a prohemosome *ph* followed by a transitional stage *t* and a hemosome *h; N* — nucleus. Bar equals 0.5 μm.

Fig. 3 — A typical hemosome *h* is seen. For comparison a mitochondrion *m* is shown in the inset; *N* — nucleus.

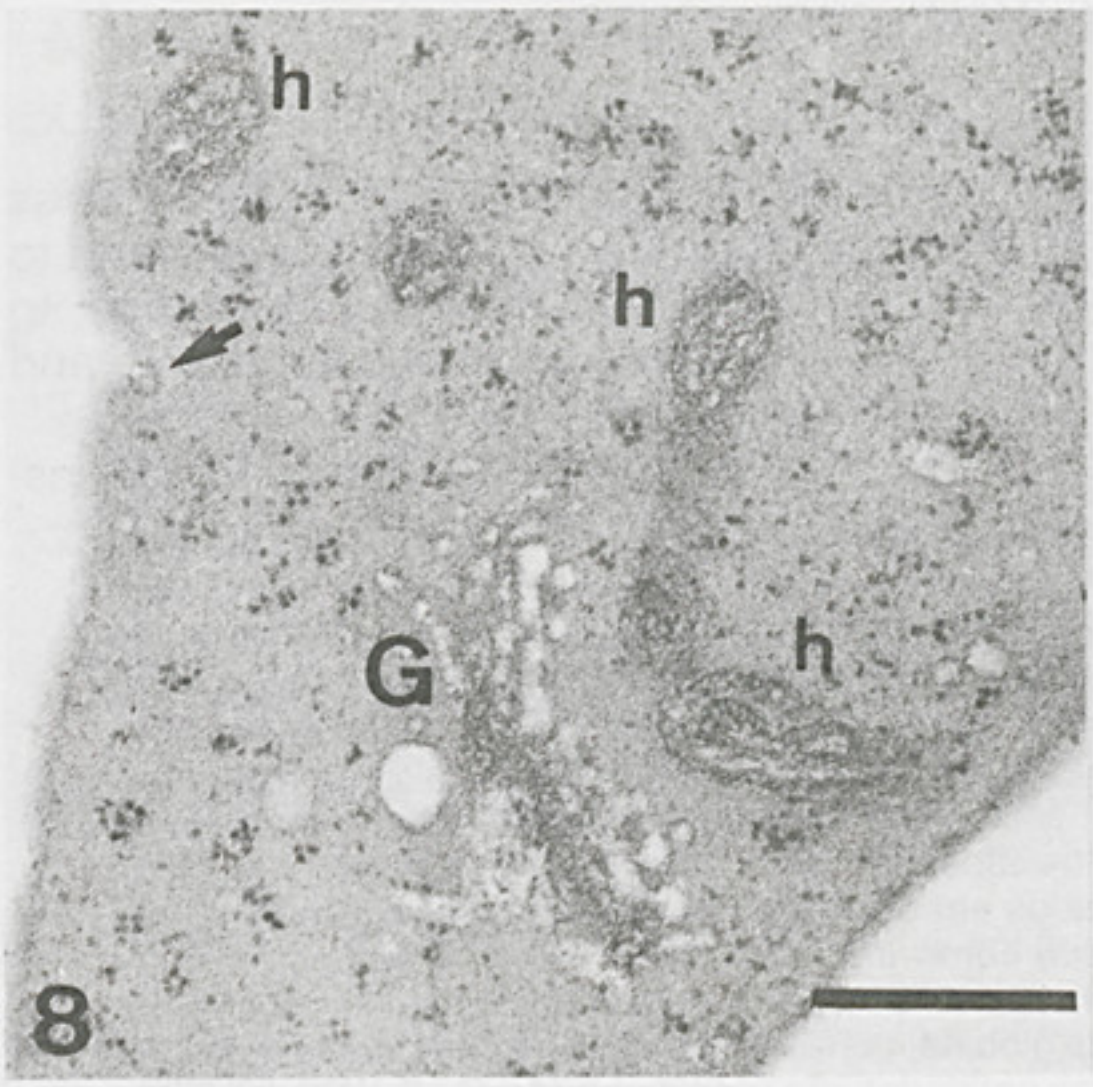
Fig. 4 — Vesicle *v* containing a still granulated ferruginous material.

Fig. 5 — A lamellated body *lb* apparently rising from a mitochondrion *m* is shown.

Fig. 6 — Condensed prehemosomal vesicle *hv* constituted by three concentric double lamellae; within the double lamellae some ribosomal particles can be observed. *Arrow* points to an endocytic vesicle.

Fig. 7 — Condensed prehemosomal vesicle *hv* modifying to prohemosomes *ph*.

Bars in Figs. 3-7 correspond to 0.25 μm.



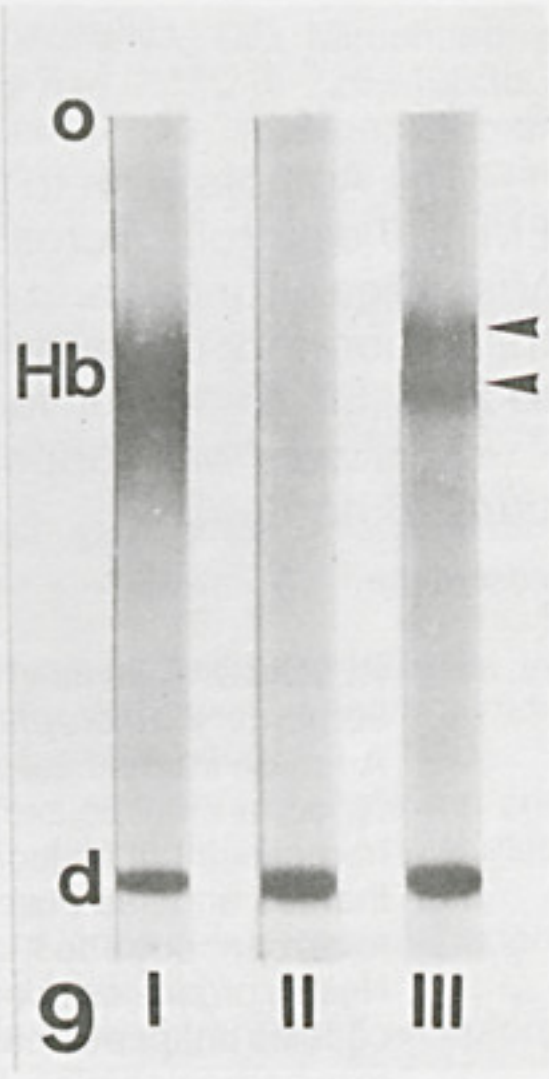


Fig. 8 — Reticulocyte showing obliquely sectioned hemosomes *h*, and a Golgi complex *G*. *Arrow* points to a rhopheocytic site. Bar equals 0.5 μm .

Fig. 9 — Electrophoretic *Hb* patterns (evidenced by tolidine) from the hemoglobinized cytoplasm (I), and from the supernatant of the lysed hemosomal fraction (III), showing two bands. The concentrated supernatant of the last washing of the fraction did not present any visible *Hb* band (II).

in the course of hemosomegenesis. According to these authors, the globin chains are enclosed in the prehemosomal vesicle, together with the ferruginous compounds, before prohemosome formation.

Typical hemosomes, showing similar structural characteristics as those found in mammalian and chicken erythroid cells, are seen in Fig. 8. The hemoglobinic nature of the hemosome content, determined through electrophoresis in polyacrylamide gel is seen in Fig. 9, showing two *Hb* bands. The hemosomal fraction isolated from erythroid cells homogenate and submitted to five washings, was osmotically lysed so that *Hb* had been released from the organelles. The supernatant of the last washing did not present any visible *Hb* band showing, therefore, that all cytoplasmic *Hb* had been discarded.

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RESUMO: Células eritróides de medula óssea e do sangue periférico da serpente *Waglerophis merremii* foram analisadas ultra-estruturalmente. A fim de intensificar a passagem de células imaturas para o sangue periférico, a eritropoiese foi estimulada através da indução de uma anemia hemolítica pela injeção de saponina. Hemossomos típicos, muito semelhantes àqueles observados em células eritróides de mamíferos e aves, foram encontrados, assim como algumas de suas formas precursoras. Nestes organelos ocorreria, provavelmente, a integração do hemo às quatro cadeias polipeptídicas de globina, considerando o seu conteúdo de natureza hemoglobínica. Estas entidades são uma derivação especializada indireta das mitocôndrias, através de sucessivas transformações para corpos lamelados, vesículas pré-hemossômicas, pró-hemossomos e, finalmente, para hemossomos.

UNITERMOS: Biossíntese de hemoglobina; hemoglobina de serpente; hemossomo de serpente.

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THE OCCURRENCE OF N-METHYLTAURINE IN THE SEA ANEMONE *BUNODOSOMA CAISSARUM*

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Akhtar HAIDER**

ABSTRACT: Significant amounts of N-Methyltaurine together with the recently described novel purine Caissarone, were isolated from the acetonic and methanolic extracts of the whole body wall of the sea anemone *Bunodosoma caissarum*. The structure of N-Methyltaurine was deduced by spectroscopic methods.
KEY WORDS: Sea anemones; Cnidaria; *Bunodosoma caissarum* Corrêa; N-methyltaurine; caissarone.

INTRODUCTION

Sea anemones belong to the *phylum* Cnidaria, one of the morphological features of which is the nematocyst or cnidocyst apparatus, also known as stinging cells, useful for prey capture. Fishes and small invertebrates are paralyzed[3] on the discharge of the nematocysts which may also inflict injurious effects on man, ranging from mild dermatitis to severe local necrosis[10]. Since toxicity is indicative of potent physiological activity, these marine organisms have attracted considerable attention because of their potential biological value. Chemical investigations have gained growing interest since the finding of tetramine, a curare-like toxin, in the tentacles of *Actinia equina*[1]. The various papers appearing over the last decades include descriptions of neuro- and cardio-active polypeptides[2], hemolytic factors[4,18], antitumoral bioassays[8,20] and organo-sulphuric[21], organo-phosphoric[14], indolic[17] and guanidinic[6] compounds.

Bunodosoma caissarum Corrêa (Anthozoa, Actiniaria) is a sea anemo-

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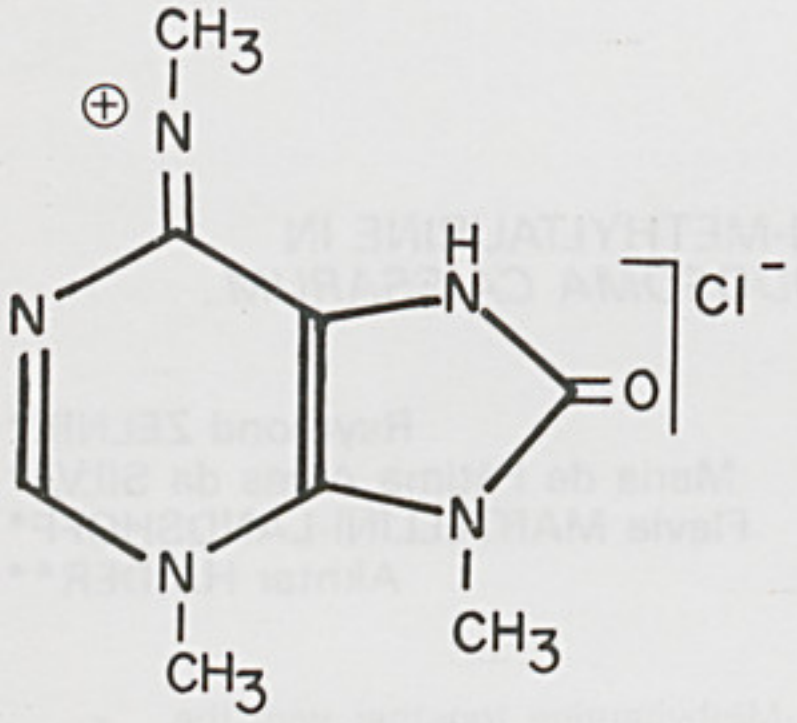
ne abundant on reefs along the Brazilian coast[7]. Material released by nematocyst discharge has been shown to be active on cholinoceptive preparations[19] and a cardiotonic polypeptide has been recovered from a methanolic extract of the anemone[13]. We have recently isolated a novel purine derivative, caissarone *1*, from the whole body walls of this cnidarian[24]. The presence of further components as revealed by thin-layer chromatography of a methanolic extract, led us to re-examine this marine organism and from which we now report the isolation of N-methyltaurine *2*.



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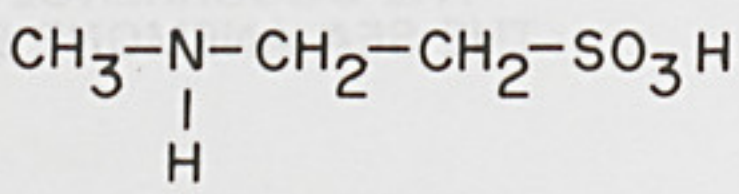

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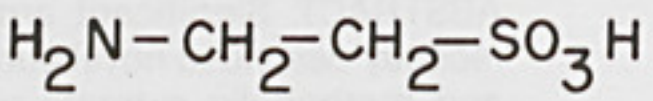


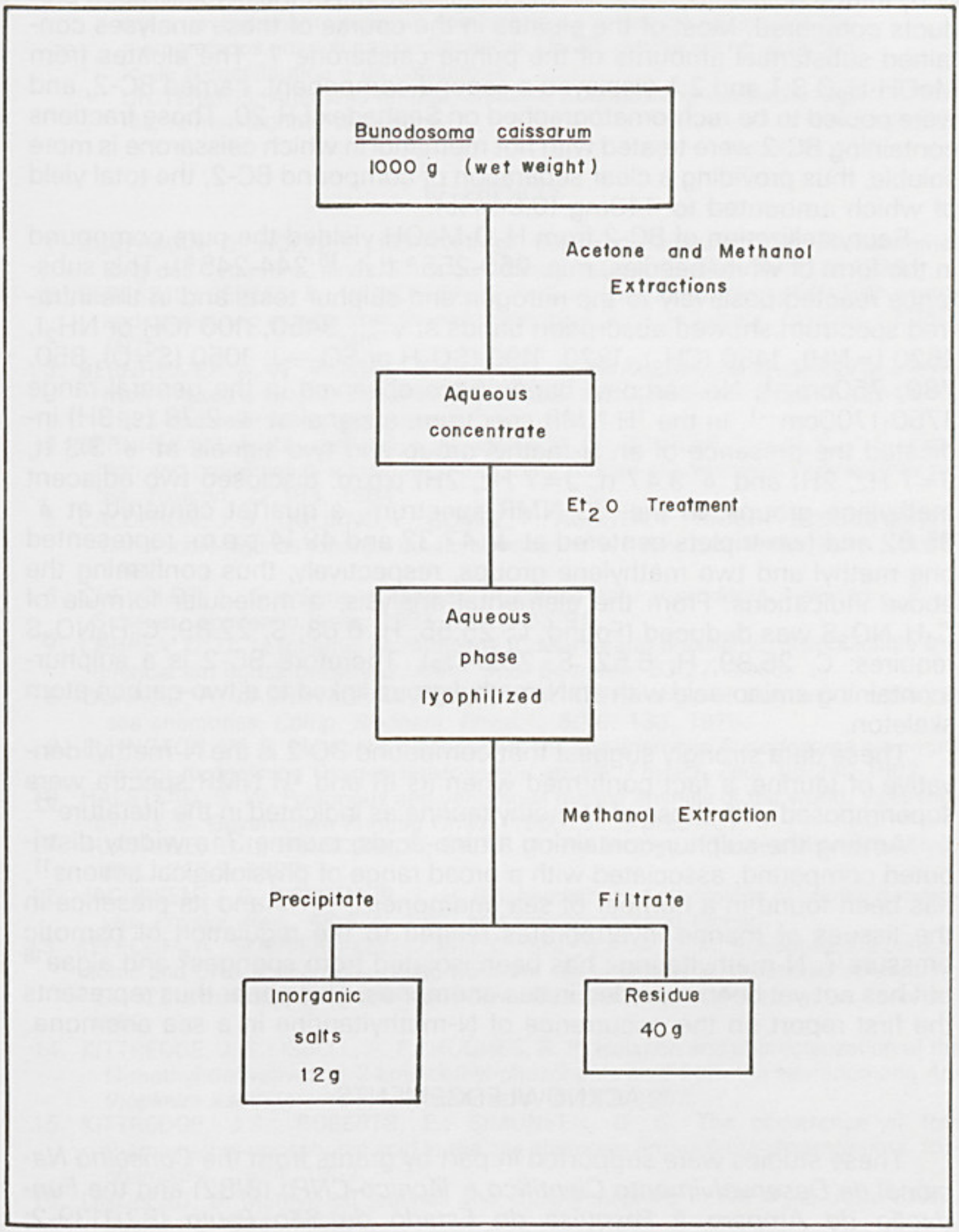
③

MATERIAL AND METHODS

Specimens were collected on the São Sebastião coast, State of São Paulo, and frozen for transport. The homogenized anemones (1.000g, wet weight) were extracted with acetone (3L) and methanol (4L) at room temperature, the extracts filtered and the combined organic phases evaporated under reduced pressure. The aqueous concentrate was then treated with diethyl ether to remove pigments, lipids and sterols and lyophilized to a viscous paste which was triturated in methanol to give a whitish precipitate (12g), containing mainly inorganic salts. The methanolic filtrate was evaporated to dryness and the residue (40g) analyzed by t.l.c. (Silica H, Merck; methanol-water 2:1; iodine vapours), revealing the presence of at least three components, one of which was readily identified as caissarone *1* by comparison with an authentic sample. Table 1 summarizes the sequence of steps used in the extraction procedure.

Melting points were determined on a Reichert-Kofler hot-stage apparatus and are uncorrected. Infra-red spectra were performed in KBr pellets, using a Perkin-Elmer 737 instrument. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker WH-360 spectrometer, operating at 360 and 90.5MHz, respectively, in D_2O solution.

TABLE 1



RESULTS

The material obtained from the last methanolic filtrate (see Table 1) was chromatographed on Sephadex LH-20 (Sigma Chemical C[a]., St. Louis, MO, USA) in batches of 3g each and the elutions were performed with graded proportions of methanol-water. The fractions were then analyzed by t.l.c.

or by infra-red spectroscopy and the melting points of the crystallization products compared. Most of the eluates in the course of these analyses contained substantial amounts of the purine caissarone *1*. The eluates from MeOH-H_2O 3:1 and 2:1 displayed a second component, named BC-2, and were pooled to be rechromatographed on Sephadex LH-20. Those fractions containing BC-2 were treated with hot methanol in which caissarone is more soluble, thus providing a clear separation of compound BC-2, the total yield of which amounted to 440mg (0.044%).

Recrystallization of BC-2 from H_2O-MeOH yielded the pure compound in the form of white needles, m.p. 250-255° (Lit. [16]:244-245°). This substance reacted positively to the nitrogen and sulphur tests and in the infra-red spectrum showed absorption bands at ν_{max} 3450,3100 (OH or NH_2), 1620 (=NH), 1480 (CH_3), 1220, 1190 (SO_3H or SO_3-), 1050 (S=O), 850, 780, 750cm^{-1}. No carbonyl bands were observed in the general range 1750-1700cm^{-1}. In the ^1H NMR spectrum, a signal at δ 2.78 (s, 3H) indicated the presence of an N-methyl group and two signals at δ 3.3 (t, J=7 Hz, 2H) and δ 3.47 (t, J=7 Hz, 2H) p.p.m. disclosed two adjacent methylene groups. In the ^{13}C NMR spectrum, a quartet centered at δ 35.82 and two triplets centered at δ 47.32 and 49.14 p.p.m. represented one methyl and two methylene groups, respectively, thus confirming the above indications. From the elemental analysis, a molecular formula of $C_3H_9NO_3S$ was deduced (Found: C, 25.55; H, 6.68; S, 22.89; $C_3H_9NO_3S$ requires: C, 25.89; H, 6.52; S, 23.04%). Therefore BC-2 is a sulphur-containing amino-acid with an N-methyl group linked to a two-carbon atom skeleton.

These data strongly suggest that compound BC-2 is the N-methyl derivative of taurine, a fact confirmed when its IR and ^1H NMR spectra were superimposed with those of N-methyltaurine as indicated in the literature[22].

Among the sulphur-containing amino-acids, taurine *3*, a widely distributed compound, associated with a broad range of physiological actions[11], has been found in a number of sea anemones[21,9,23,15] and its presence in the tissues of marine invertebrates related to the regulation of osmotic pressure[12]. N-methyltaurine has been isolated from sponges[5] and algae[16] but has not yet been recorded in sea anemones. This paper thus represents the first report on the occurrence of N-methyltaurine in a sea anemone.

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ZELNIK, R.; SILVA, M.F.A. da; MARTELLINI-LANDSHOFF. F.; HAIDER A. The occurrence of N Methyltaurine in the sea anemone **Bunodosoma caissarum. Mem. Inst. Butantan, 51**(4):141-146, 1989.

RESUMO: A anêmona-do-mar *Bunodosoma caissarum* Corrêa foi submetida a um tratamento extrativo com acetona e metanol e os extratos cromatografados em colunas de Sephadex LH-20, resultando no isolamento de N-metiltaurina e de caissarone.
UNITERMOS: Anêmona-do-mar; cnidaria; *Bunodosoma caissarum* Corrêa; N-metiltaurina; caissarone.

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STANDARDIZATION OF ANESTHESIA WITH PENTOBARBITAL IN THE SNAKE *BOTHROPS JARARACA**

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Marília C.L. SEELAENDER**
Eva M.A. KELEN**

ABSTRACT: A standardized surgical anesthesia was established for *Bothrops jararaca* snakes injected subcutaneously with 30 mg/kg of pentobarbital *(Nembutal)*. Induction times ranged between 30 to 40 minutes for 63 of the 79 injected snakes (79,7%).
These uniform results are different from those obtained with intrapleuroperitoneal injections (17 snakes), currently used.
KEY WORDS: Subcutaneous anesthesia, anesthesia in snakes with pentobarbital, *Bothrops jararaca* anesthesia.

INTRODUCTION

A standardization of anesthesia during experiments with the Crotalidae snake *Bothrops jararaca* was required for surgical procedures. A long-lasting, stable state of anesthesia and a fast induction time were needed.

Inconstant results are reported for anesthesia after intrapleuroperitoneal (IPP) or intramuscular (IM) injections of pentobarbital in different snakes[1,2]. Karlstrom and Cook[5] suggested that results suffer the influence of factors as size, individual physiology, condition of the snake at the time of injection, room temperature, site of injection and degree of handling of the animal.

The subcutaneous (SC) route for pentobarbital administration has not been reported routinely, although it has been used for anesthesia with an ultrashort-acting barbiturate, Brevital Sodium[6] and with Ketamine Hydrochloride[4]. The SC injection of Brevital Sodium lead to a short induction time and a short-lasting anesthesia, which allowed surgical procedures

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when 15 mg/kg body weight were administered in *Thamnophis sirtalis* and *Natrix sipedon sipedon*[6].

The efficiency of the subcutaneous administration of pentobarbital for the anesthesia of *Bothrops jararaca* snakes is here reported.

MATERIAL AND METHODS

The anesthetic employed was Sodium Pentobarbital *(Nembutal,* Abbott Laboratories) as a 5% solution prepared from capsules containing 100mg. The salt was dissolved in distilled water with the help of a magnetic stirrer and stored at 4°C after centrifugation. *Bothrops jararaca* snakes, 42 males (70 to 400g, mean of 154g) and 54 females (90 to 655g, mean of 260g), received each, an optimal dose which was standardized for these snakes as 30mg/kg body weight, either subcutaneously or intrapleuroperitoneally. The administration of the anesthetic was performed while an operator immobilized the snake by holding head and tail, and a second operator made the injection. IPP injections (17 snakes) were made ventrally, into the distal third of the body, avoiding the pericardial region. The SC injections (79 snakes) were made at the junction of ventral and dorsal scales, approximately at 10cm below the heart. The presence of a gap, felt with the needle, indicates the space between skin and muscle to be injected.

Three main stages of anesthesia were arbitrarily established:

I-Sedation: slow movements as response to pressure of the tail with a hook. Presence of righting reflex. Normal size pupils, easily distinguished in the light brown colored eyes of this snake.

II-Partial anesthesia: incapacity of promoting serpentine movements. Loss of righting reflex. Tail-withdrawal reflex on pressure still present. Semi-contracted pupils.

III-Deep anesthesia: loss of tail-withdrawal reflex, lack of any sort of response to pressure of the hook. Contracted pupils.

The stage of anesthesia required was stage III which lasted for a minimum of three hours. Experiments were carried out at different ambient temperature ranges. No artificial respiration was needed. The snakes were not fed over a period of, at least, 96 hours before anesthesia.

RESULTS

Table 1 indicates the number of anesthetized snakes related to time (minutes) of anesthesia induction at the level of stage III. Results are expressed in ten minutes intervals. Among the 79 subcutaneously injected snakes, 63, weighing from 70 to 655 g, were anesthetized within the range of 30 to 40 minutes (79,7%). The stage of anestesia was demonstrated to be stable and no supplementation of dose was ever necessary. The effect of the anesthetic lasted for at least three hours but the exact time of recovery was not observed with the exception of two animals, which recovered within 48 hours. None of the 79 animals died under this treatment. The stage of recovery from anesthesia was considered when the snake was back to all the reflexes affected during anesthesia (stages I through III).

Among the 17 intrapleuroperitoneally injected snakes, increasing times of induction were directly proportional to increasing body weight. For ex-

ample, 3 snakes, weighing 106 to 127 g were anesthetized after 30 minutes, while 4 others, weighing 161 to 400g reached stage III within 50 minutes. Stage III suffered oscilations in three animals. Recovery took 23 to 48 hours and 5 of the snakes died before recovery. Figures 1 and 2 illustrate the different behaviour of subcutaneously and intrapleuroperitoneally injected *Bothrops jararaca* snakes, in relation to the time of induction of stage III.

TABLE 1

Anesthesia of *Bothrops jararaca* snakes with pentobarbital. Time of induction to the level of stage III in 10 minutes intervals after intrapleuroperitoneal (IPP) or subcutaneous (SC) injections of a 5% anesthetic solution. Dose: 30 mg/kg body weight.

	Induction time of stage III minutes	Number of snakes	Body weight g	
			mean	range
	10	00	—	—
	20	04	360	206-564
	30	39	179	75-353
	40	24	288	70-655
SC	50	09	196	120-335
	60	00	—	—
	70	02	415	345-485
	80	00	—	—
	90	01	369	369
	no induction	00	—	—
	10	00	—	—
	20	00	—	—
	30	03	118	106-127
	40	02	195	140-250
IPP	50	04	288	161-400
	60	02*	253	200-305
	70	01	405	405
	80	00	—	—
	90	01*	431	431
	no induction	04	272	204-445

* Oscilation of stage III

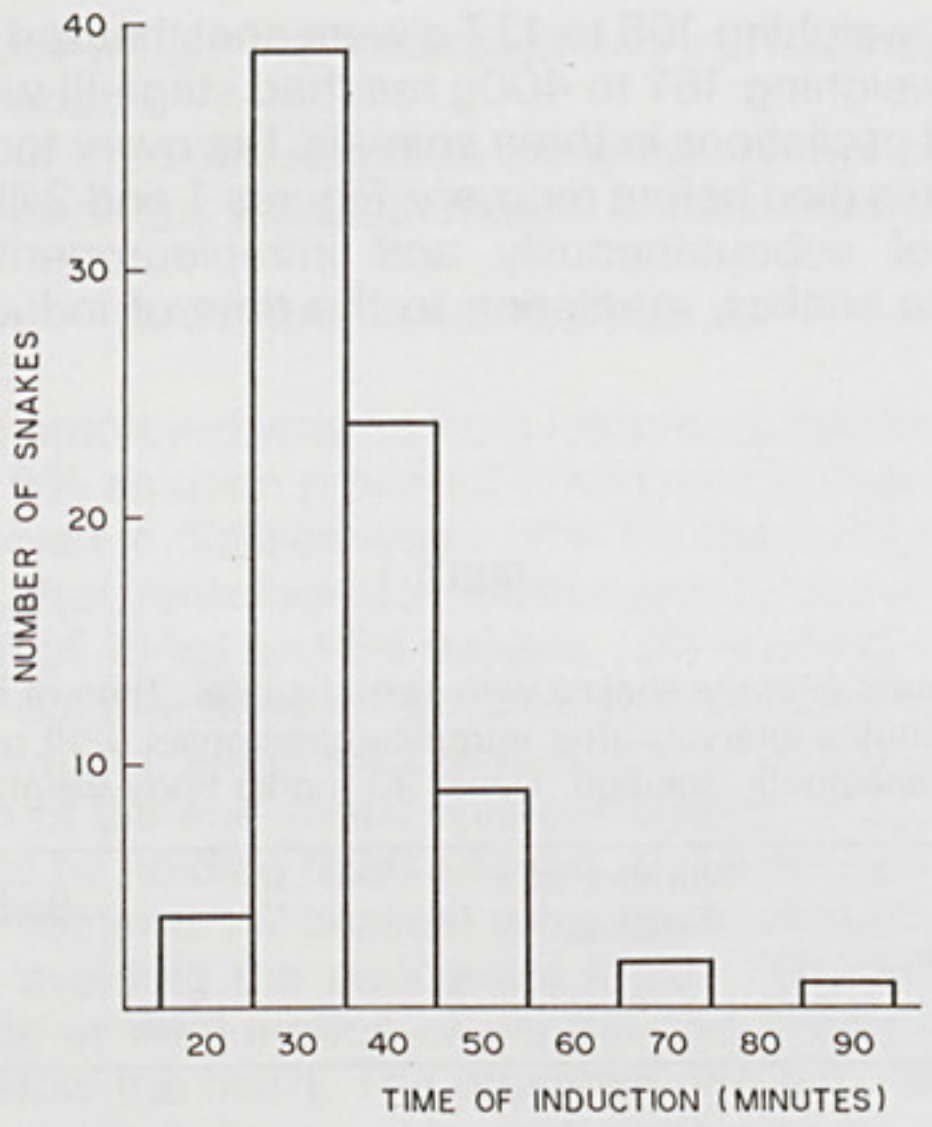

FIG. 1: Distribution of *Bothrops jararaca* snakes according to time of induction of deep anesthesia (stage III) in 10 minutes interval, after subcutaneous injections of pentobarbital (30 mg/kg body weight).

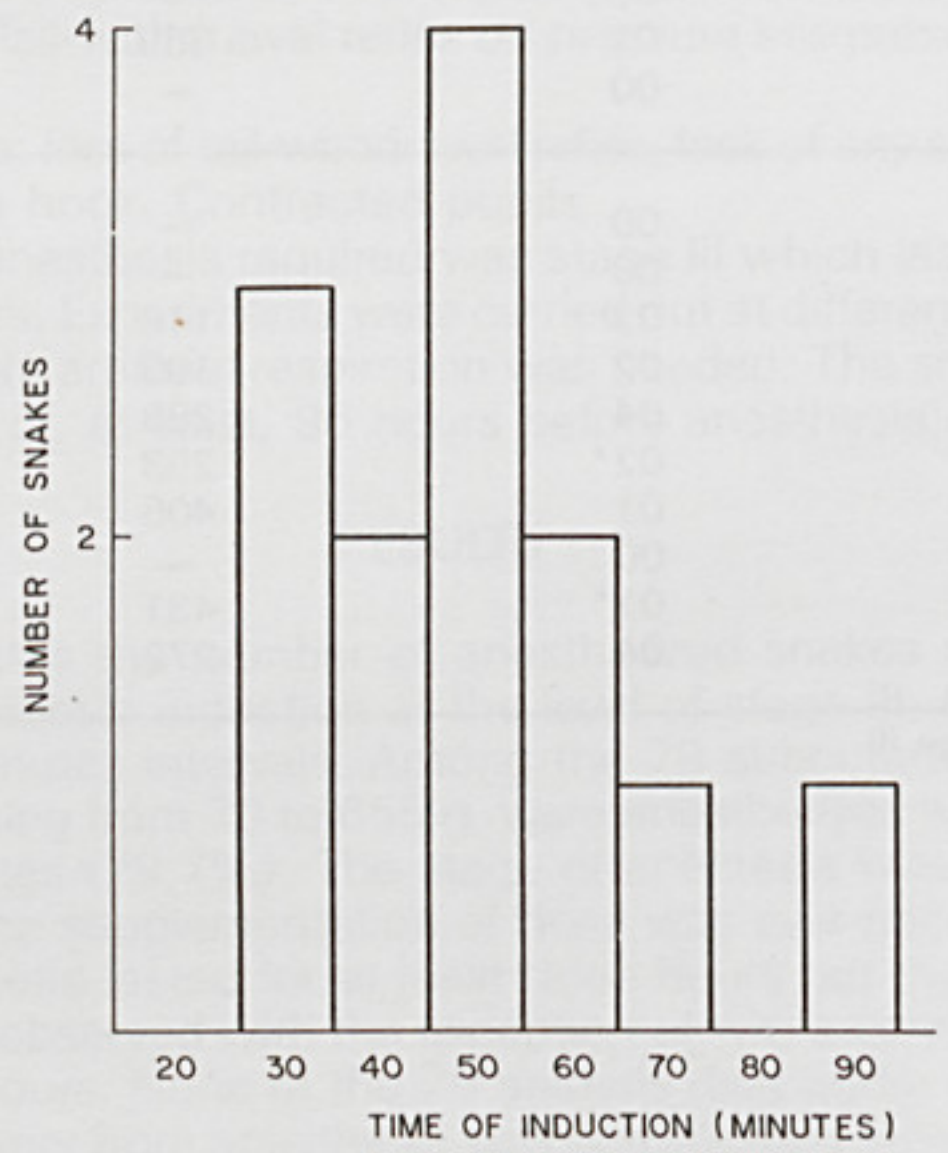

FIG. 2: Distribution of *Bothrops jararaca* snakes according to time of induction of deep anesthesia (stage III) in 10 minutes interval, after intrapleuroperitoneal injections of pentobarbital (30mg/kg body weight).

DISCUSSION

Results demonstrate that a standard dose of the anesthetic *(Nembutal)* per unit of body weight, can be used with no need for supplementation when SC administration is performed in *Bothrops jararaca* snakes. According to these results, about 80% of the snakes reached stage III of anesthesia within 30 to 40 minutes, their body weight ranging from 70 to 655 g. Experiments were performed at different seasons, when the temperature variation was from 3.5 to 34.6°C. From this, it is evident that subcutaneously induced anesthesia with pentobarbital is not influenced by size variation or by ambient temperature modification during the procedure.

The criteria of stages arbitrarily established to follow intensity degrees was a helpful step for the standardization procedure. It was also used by Genevois *et al*[3]. with slightly different definitions.

The results obtained with IPP injections of pentobarbital in 17 *Bothrops jararaca* corroborate the data from literature, concerning the influence of weight variation in snakes under a same anesthetic dose, since larger ones had an elongated induction time as demonstrated in table 1.

The possibility that the intense integumentary vascularization in snakes[7], in which circulation is modified during thermoregulation[8], might be involved in the regular absorption of the anesthetic by the SC route should be further investigated. Inconsistent results in the IPP induced anesthesia may be due to difficulties in defining the site and the depth of injection, in view of the extense and not well delimited pleuroperitoneal cavity, in which the diaphragm is lacking. The risk of organ injury must be also mentioned.

The standardization obtained for *Bothrops jararaca* with the SC route of injection of *Nembutal* indicates this administration as extremely convenient when deep anesthesia is required.

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Thanks are due to Augusto S. Abe, State University of São Paulo, Campus of Rio Claro-SP, and Andreas Moser, Visiting Biologist from Basel, Switzerland, for fruitful discussion. A.M.C. was recipient of grant no.TB/1439-80 from FEDIB at the time of the standardization experiments. M.C.L.S. was under a scholarship from FAPESP (nº 86/3057-4). The skillful technical assistance of Jorge Abel Ferreira in the contention of animals is gratefully acknowledged as well.

RESUMO: Foi obtido um método padronizado de anestesia cirúrgica para serpentes *Bothrops jararaca,* injetadas com 30mg/kg de pentobarbital (Nembutal) por via subcutânea. O tempo de indução variou entre 30 e 40 minutos para 63 de 79 serpentes estudadas (79,7%). Estes resultados uniformes contrastam com os obtidos com a via intrapleuroperitoneal (17 serpentes), normalmente adotada.
UNITERMOS: Anestesia subcutânea, anestesia em serpentes com pentobarbital, *Bothrops jararaca* anestesia.

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CROSS-REACTIVITY OF HORSE MONOVALENT ANTIVENOMS TO VENOMS OF TEN *BOTHROPS* SPECIES

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ABSTRACT: Horses were immunized with *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* venoms. Antibodies recognizing the venom antigenic components were either immunochemically detected by the enzyme-linked immunosorbent, double immunodiffusion and quantitative precipitation methods or biologically by the assays measuring the venoms indirect hemolytic and lethal toxic activities. Specific and cross-reacting antibodies against the *Bothrops* venoms were found in all ten monovalent antivenoms. Modifications in the serum electrophoretic patterns characterized by a reduction of the albumin peak and by a correspondent increase of the γ-globulins with a patent or no modification of the α or β globulins were found in these sera.
KEY WORDS: Antivenoms, *Bothrops* venoms, monovalent *Bothrops* antivenoms, cross-reactivity reactions.

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The "Comissão de Soros, Ministério da Saúde, Brasil", has recommended studies to determine the satisfactory *Bothrops* venoms mixture to be used as antigen to produce the corresponding antivenom. Therefore, a study group at the "Instituto Butantan, São Paulo, Brasil" was convened and charged with these studies and this paper contains the result obtained.
Dedicated to Dr. Saul Schenberg's 70th birthday.
Received 8/5/1989; accepted 18/7/1989.

INTRODUCTION

Horse antivenoms against *Bothrops* venoms have been produced using as antigen a mixture composed of venoms from seven of this snake species. At least ten well characterized snake species, belonging to the genus *Bothrops*, have been identified in Brazil and it is well known that the venoms from these snakes as complex mixtures, contain various enzymes in addition to toxic elements. There are evidences that some of these components are largely distributed among the majority whereas others are restricted to some species. This raises questions concerning the ability of antisera routinely produced for therapeutic purposes to neutralize the relevant components responsible for the pathophysiologic action of the venom as a whole. In order to clarify these practical posed questions two experimental protocols were delineated: a) production of monovalent antisera against each of the ten *Bothrops* venoms and analysis for the presence of antibodies against the components found both in the venom used for immunization and in the venoms from the other *Bothrops* species; and, b) to test in parallel assays, the polyvalent *Bothrops* antivenoms routinely prepared in the Instituto Butantan for therapeutic purposes for their capacity to combine with the components present in each individual *Bothrops* venom.

ABBREVIATIONS

LD_{50}.....lethal dose 50%
BVM.....*Bothrops* venoms mixture
FCA.....Freund's complete adjuvant
FIA.....Freund incomplete adjuvant
PBS.....phosphate saline buffer
BSA.....bovine serum albumin
NPGB.....p-nitrophenyl-p' guanidino benzoate
PMSF.....phenyl-methyl sulphonyl-phluoride
EDTA.....ethylene-diamine tetraacetic acid

MATERIAL AND METHODS

Animals. Adult horses with 400-450kg of body weight maintained at the São Joaquim farm, Instituto Butantan were to produce both, the monovalent and the *Bothrops* polyvalent antivenoms. Outbred albino mice weighing 18-22 g from Biotério Central, Instituto Butantan were used for the neutralization test.

Venoms. Bothrops venoms from the *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* were pooled venoms from several adult snakes maintained in the "Seção de Venenos" of the Instituto Butantan. Venoms were extracted, desiccated and stored at 4°C before use. Stock solutions of venoms from each *Bothrops* species were, as otherwise indicated, prepared by diluting 100mg of dried venom in 10 ml of 0.15M phosphate buffer at pH 7.2. The *Bothrops* venom mixture used to produce the polyvalent antivenoms was prepared by adding one volume 10mg/ml solution of *B. jararaca* venom to an equal volume of a 10 mg/ml solution containing equal parts

of venoms from *B. alternatus, B. neuwiedi, B. jararacussu, B. cotiara, B. moojeni* and *B. pradoi*. The immunizing mixtures were prepared just before use, by diluting stock solutions to a final concentration of 5 mg/ml in: a) Freund's complete adjuvant (FCA); b) Freund's incomplete adjuvant (FIA); c) alginate; d) 0.15 M NaCl. The LD_{50} values for each *Bothrops* species were previously determined by the ''Seção de Controle'' of the Instituto Butantan by the probit analysis Finney[4]. The values for each *Bothrops* species are indicated in parenthesis: *B. alternatus* (66.0 μg); *B. atrox* (145.0 μg); *B. cotiara* (46.79 μg); *B. erythromelas* (70.0 μg); *B. insularis* (50.0 μg); *B. jararaca* (33,0 μg); *B. jararacussu* (76.0 μg); *B. moojeni* (115.2 μg); *B. neuwiedi* (35.5 μg) and *B. pradoi* (61.56 μg).

Antivenom production. Monovalent antivenoms were obtained from horses subcutaneously injected with one *Bothrops* venom each: *B. alternatus* (horse nº 120), *B. atrox* (horse nº 144), *B. cotiara* (horse nº 134), *B. erythromelas* (horse nº 25 and 37), *B. insularis* (horse nº 54 and 154), *B. jararaca* (horse nº 141), *B. jararacussu* (horse nº 162), *B. moojeni* (horse nº 290), *B. neuwiedi* (horse nº 980) and *B. pradoi* (horse nº 131). The animals were previously sensitized by a primary stimulus with 5.0 mg of the venoms in FCA. Six months later horses were reinjected subcutaneously with 3 mg of the corresponding venom in 10 ml of alginate. After three months a third reinjection was performed with 5 mg venom in FIA followed by three injections of 2,5mg venom in 0.15 M NaCl, at 7 days intervals. Seven liters of blood were collected one week later and the plasma separated and stored at 4°C. Polyvalent plasma with antibodies, against the mixture of venoms from *B. alternatus, B. cotiara, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* were obtained by immunizing horses with BVM essentially by the same immunization schedule.

Purification of horse IgG and its F(ab')$_2$ fragments. All purifications were carried out using the same pool horse plasma.

IgG. IgG was prepared as described by Steinbuch and Audran[14]. Horse hyperimmune plasma at a concentration of 70 mg/ml, was heated at 56°C for 15 min and subsequently centrifuged at 900 x g for 10 min. The pH of the supernatants was adjusted to 5.0 with 0.1 N acetic acid and caprylic acid (Merck, Darmstadt) was then added, to a final concentration of 8.7%, under vigorous stirring for 30 min at room temperature. After centrifugation (15 min, 1000 x g) the supernatant was filtered through a 0,45 μm Millipore membrane (Millipore Corporation, Bedford, MA 01730), and dialysed overnight against 0.85% NaCl at 4°C. The IgG preparation was stored at -20°C. *F(ab')$_2$. The method of Pape[12] Slightly modified was used to obtain F(ab')$_2$ fragments from both monovalent or polyvalent horse hyperimmune plasma according to the procedures established by the Instituto Butantan (unpublished data): Briefly, Bothrops* antivenom plasma diluted to 40 mg/ml with distilled water was heated at 30°C. The pH was adjusted to 3.1 with 2.5 M HCl, and pepsin (INLAB, São Paulo, Brazil) was then added under slow stirring (5.0 gr per liter of plasma). The mixture was incubated for 40 min at 30°C, followed by the addition of tetrasodium pyrophosphate (Labsynth, São Paulo, Brazil) (0.1% final concentration) and toluene (INLAB, São Paulo, Brazil) (0.1% final concentration). Temperature was then elevated to 55°C and the pH was adjusted to 5.2 with 20% NaOH. After adding ammonium sulphate (Quimis, São Paulo, Brazil) to a final concentration of

17.5%, the solution was stirred during 90 min at 55°C and then centrifuged for 10 min at 900 x g. The supernatant was removed and the pH adjusted to 6.9-7.1 with NaOH. Ammonium sulphate was again added to the mixture to a final concentration of 28% which was stirred at room temperature for 60 min. After centrifugation, the precipitate was resuspended in 0.85% NaCl and the resulting solution dialysed for 48h against 0.85% NaCl at 4°C. The $F(ab')_2$ rich preparation was stored at -20°C. The protein content was measured by the method of Lowry *et al.*[9] using bovine serum albumin as a standard. The preparations obtained were termed monovalent or polyvalent *Bothrops* antivenoms.

The ELISA method Theakston et al.[15] One hundred ul of *Bothrops* venoms (1 μg/ml) were absorbed to the wells of Nuclon plates (Delta, Denmark), at 4°C, overnight, blocked with 3% BSA in phosphate buffered saline containing 0.05% Tween 20 for 3h at room temperature. The plates were washed with 0.05% of BSA in phosphate buffered saline containing 0.05% of Tween 20. One hundred μl of several dilutions of each serum sample (1/100-1/12,000) were added to the wells and allowed to incubate for 45 min at room temperature. The plates were washed again as mentioned and 100 μl of peroxidase conjugated anti-mouse immunoglobulin diluted 1/1,000 (Cappel, Cochanville, Pa) were added to each well. Plates were incubated for 45 min at room temperature. After washing the wells as previously described, 100 μl of ortho-phenylenediamine (Sigma Co., U.S.A) (1 mg/ml) and 4 μl of H_2O_2 were added to wells and plates were allowed to stand at room temperature for 15 — 20 min before spectrophotometric determination of colour change.

Double Immunodiffusion Ouchterlony[11]. Agarose was dissolved in 0.056 M sodium veronal buffer, pH 8.6, at 0.8% concentration and overlaid on glass plates. The plates contained one center well and eight outer wells (3mm in diameter) at 4mm distance. Twenty microlliters of *Bothrops* crude venoms were added to central wells and tested against eight dilutions of the corresponding horse antisera pipetted in the outer wells. The plates were allowed to develop for 24h at room temperature and stained with Coomassie blue. Antivenoms were arbitrarily classified as weak reactors (WR) and strong reactors (SR) according to the presence of precipitin bands at dilutions up to 1:64 and 1:128, respectively.

Cellulose acetate electrophoresis. Electrophoresis of horse sera obtained immediately before and after the immunization with each *Bothrops* venom was performed in 0.04M sodium veronal buffer pH 8.6 during 25 minutes at 200 Volts. Strips were stained for 8 minutes in 0.5% amidoblack 10B dissolved in a mixture of 47.5% methanol and 5% acetic acid, and destained in the same mixture. They were dried in absolute methanol, and treated with a solution containing 85% methanol, 14% acetic acid, 1% glycerol. Absorbance was determined in a densitometer. In control samples the typical plasma protein bands were clearly seen: albumin and the globulins $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$ and $\gamma 2$.

Ability of Bothrops antivenoms to neutralize the indirect hemolytic activity of Bothrops venoms. 0.3 ml of packed sheep erythrocytes were washed four times with 0.15 M NaCl and pelleted. To these pellets were added 0.3 ml of egg yolk diluted 1:4 in 0.1M NaCl containing 0.25 ml of 0.01 M $CaCl_2$, resuspended and mixed with 25 ml of 0.8% agarose dissolved in phosphate-

buffered saline solution, pH 8.1. 18 ml of this solution were overleyed on 40 x 180 mm glass plates and allowed to solidify. 4mm diameter wells were punched in the middle of the plates 40mm apart and filled either with 15 μl of a standard solution of venoms previously standardized to give hemolytic haloes of 20 mm or a mixture containing these amounts of venom plus the antivenoms at different dilutions. The plates were incubated at 37°C for 24h, the hemolytic haloes measured and the percentage of inhibition was calculated.

Quantitative precipitin reaction Heidelberger[6]. The IgG immunoglobulins used were obtained from the different *Bothrops* monovalent antivenoms purified with aid of caprylic acid and the corresponding *Bothrops* venoms were used as the source of antibody and antigen, respectively. The IgG preparations containing 1 mg protein/ml were dissolved in phosphate-saline buffer (PBS) pH 7,4 and diluted 1:4 before use. In order to minimize the possible IgG cleavage by the proteolytic enzymes present in the *Bothrops* venoms this buffer contained 5 μM p-nitrophenyl — p'-guanidino benzoate (NPGB), 5 μM phenyl-methyl sulphonyl-phluoride (PMSF) and 10mM ethylenediamine tetracetic acid (EDTA). To a series of tubes (13 x 70 mm) were added 0.5 ml of IgG solution and 0.5 ml of the corresponding *Bothrops* venom solution containing, 20, 40, 80, 160, 320, 640, 1,000 and 1,280 μg, mixed and allowed to stand overnight at 4°C. Controls containing IgG or the highest amount of venom alone were rumning in parallel. The precipitates were washed three times with cold PBS, ressuspended in 0.1 N NaOH and the total protein contents determined by the Lowry's method (1951)[9].Knowing the amounts of antigen (Ag) added in each tube, the corresponding values for antibody (Ab) and the ratios Ab:Ag were calculated. Graphics were constructed by plotting the Ag values on ordinates (Y) and the ratios Ab:Ag (X) on abscissae. A straight line was obtained following the equation $\frac{Ab}{X} = a - bx$, or $Ab = a - b(x)^2$ in whic "x" is the amount of Ag added and the "a" and "b" constants representing the intercept of the line on the "Y" axis and "slope", respectively. Using these values graphicaly obtained and the values for the known maximal Ag precipitated as substitutes for "a", "b" and "X", respectively, in the above derived equation, the maximal values for specific Ab in each monovalent IgG preparation were obtained. As the venoms are complex mixtures of several immunogenic components and the correspondent antisera shall have antibodies specific for distinct epitopes and antigens, the precipitin curves obtained in each venom-antivenom assay was logistically transformed in order to determine their parallelism and coincidence. The values for Ab on the precipitin curves were applied to the equation $y = 1n.\frac{Ab/2,000}{1-Ab/2,000}$, Bussab[2].

The linear function y=A (μg of venom) + B was obtained where A is the angle coeficient and B the linear coeficient, these values being calculated by the "SAS square minimal program Ray[13]. The parallelism and the coincidence among the retified curves were determined by the F test Choi[3], according to the criteria used by Murata[10] in their analyses of horse anti-*Crotalus durissus terrificus* venom when assayed against gamma radiated venom.

Mouse protection test. This assay was performed strictly obeying the following conditions and methodology:

a) Outbred albino mice with 18-22g body weight were used.

b) The venom solutions containing 5 LD_{50} were incubated with an equal volume of different antivenom dilutions (dilution factor of 1.2) at 37°C for 30 min. Control mixtures containing 0.15M NaCl instead of antivenom were running in parallel. Eight mice per dilution point or for the control were intraperitoneally injected with 0.5 ml of each solution, the animals being observed for 48h and the number of alive animals recorded. The probits were calculated with the aid of the percentage of survival values obtained for each serum dilution, plotted on a semi-log paper (in abscissae) against the volume of antivenom (in ordenates) and the 50% mortality determined. With the aid of the formule TV-1 in which TV = 5 LD_{50} in μg, the amount of venom in mg was calculated.

RESULTS

Antibodies detected by the ELISA assay.

According to their reactivity the *Bothrops* monovalent antivenoms were classified for descriptive purposes as weak reactors (WR), intermediate reactors (IR) and strong reactors (SR), with antibody titers below 64 x 10^3, up to 128 x 10^3 and over this latter values, respectively. From the data presented in Table 1 three observations can be drawn: a) *B. cotiara* venom produces WR antisera against all venoms; b) *B. alternatus* and *B. insularis* venoms produce SR antisera either when tested against the specific venom or in cross-testing one against each other; and c) the remaining venoms produce IR or WR antisera both in specific and in cross-testing assays.

Antibodies detected by the double diffusion method.

The *Bothrops* monovalent antivenoms were also classified as WR (titers up to 64) and SR (titers over 128) according to their maximal dilution giving visible precipitin bands. Table 2 shows: a) *B. cotiara* produced SR antiserum against all venoms; b) *B. alternatus, B. insularis, B. jararaca, B. moojeni, B. neuwiedi* and *B. pradoi* produced SR antisera at least when tested against one of the *Bothrops* venoms; and, c) *B. alternatus, B. atrox, B. erythromelas* and *B. jararacussu* venoms produced WR antisera.

Electrophoretic profiles of horse **Bothrops** *antivenoms.*

In comparison with normal horse serum the relative quantities of the serum proteins in *Bothrops* antivenoms as determined by electrophoresis on cellulose acetate strips were the following:

a) Albumin was unequivocally reduced in *B. atrox, B. moojeni* and in BVM antivenoms but only slightly reduced in *B. cotiara, B. insularis, B. jararaca, B. jararacussu* and *B. pradoi* or unchanged in *B. alternatus* and *B. neuwiedi* antivenoms; b) excepting for *B. cotiara, B. insularis* and *B. moojeni*, α -globulins were decreased in the (ileg.) *Bothrops* antivenoms; c) the β --globulins, excepting for *B. atrox, B. cotiara, B. jararaca, B. pradoi* and BVM antivenoms, β-globulins were reduced; and, d) the γ-globulins, excepting for the *B. erythromelas* antivenom, were increased in the other *Bothrops* antivenoms. Table 3 and Fig. 1 show these results.

TABLE 1

Antibody production in horses immunized with different *Bothrops* venoms, measured by the enzyme linked immuno-absorbent assay (ELISA) and classified as strong (SR), intermediate (IR) and weak reactors (WR)[a]

Bothrops antivenoms		*Bothrops* Venoms									
		alt	atr	cot	ery	ins	jar	jsu	moo	neu	pra
B. alternatus	(alt)	SR	WR	WR	WR	SR	WR	IR	IR	WR	IR
B. atrox	(atr)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. cotiara	(cot)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. erythromelas	(ery)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. insularis	(ins)	SR	WR	WR	WR	SR	WR	WR	WR	WR	WR
B. jararaca	(jar)	WR	WR	WR	WR	WR	IR	IR	IR	IR	IR
B. jararacussu	(jsu)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. moojeni	(moo)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. neuwiedi	(neu)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. pradoi	(pra)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR

[a]WR, titers of 64 x 10^3; IR, titers of 64 x 10^3 to 128 x 10^3; SR, titers over 128 x 10^3

TABLE 2

Cross-reactivity of *Bothrops* antivenoms with different *Bothrops* venoms as determined by double immuno-diffusion and classified accordingly as strong reactor (SR) and weak reactors (WR)[a]

Bothrops antivenoms		Bothrops Venoms									
		alt	atr	cot	ery	ins	jar	jsu	moo	neu	pra
B. alternatus	(alt)	WR	WR	SR	SR	WR	WR	WR	SR	WR	WR
B. atrox	(atr)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. cotiara	(cot)	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR
B. erythromelas	(ery)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. insularis	(ins)	SR	WR	WR	WR	WR	SR	SR	WR	WR	WR
B. jararaca	(jar)	SR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. jararacussu	(jsu)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. moojeni	(moo)	SR	SR	WR	WR	SR	WR	SR	WR	WR	SR
B. neuwiedi	(neu)	SR	WR	WR	WR	WR	WR	SR	WR	WR	WR
B. pradoi	(pra)	WR	SR	WR	WR	WR	WR	WR	WR	WR	WR

[a]Strong reactors (SR): titers of 128 and over; weak reactors (WR): titers up to 64.

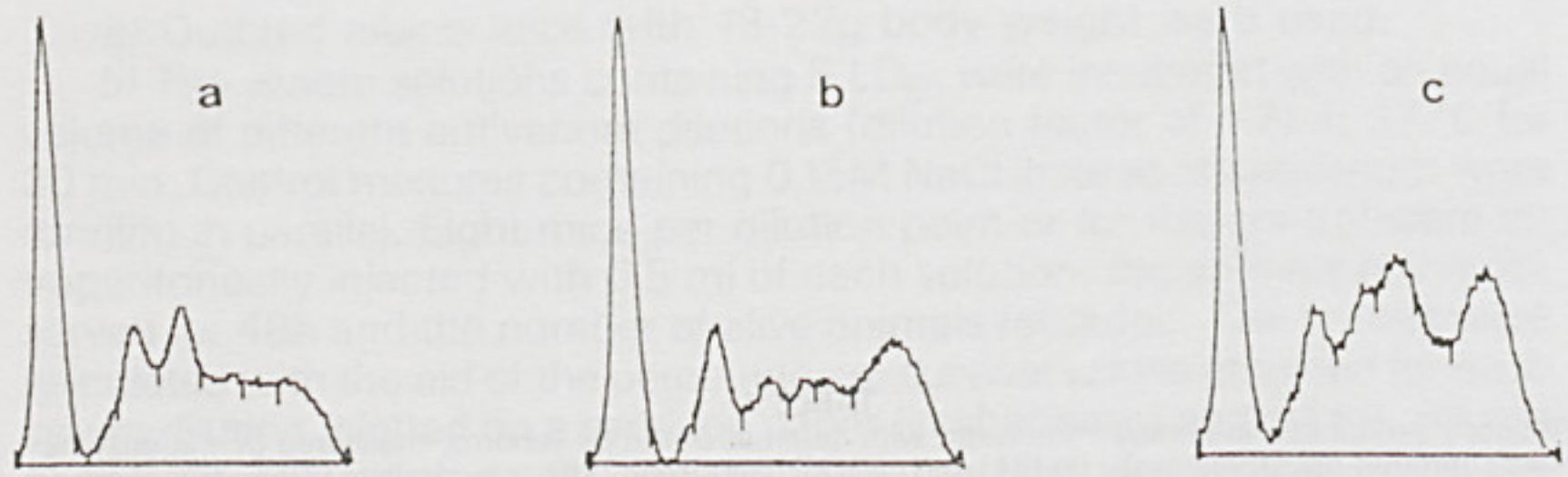

Fig. 1: Cellulose acetate electrophoresis of sera obtained from normal (panel a) or from hyperimmunized horses with venoms of *B. alternatus* (panel b), *B. atrox* (panel c), *B. cotiara* (panel d), *B. erythromelas* (panel d), *B. insularis* (panel e), *B. jararaca* (panel f), *B. jararacussu* (panel g), *B. moojeni* (panel i), *B. neuwiedi* (panel j), *B. pradoi* (panel l) and the mixture of *Bothrops* venoms composed of 50% of a solution containing *B. jararaca* venom and 50% of a solution containing equal parts of venoms from *B. alternatus, B. cotiara, B. jararaca, B. moojeni, B. neuwiedi* and *B. pradoi*.
Samples of horse sera were applied on cellulose acetate strips and electrophoresed in 0.04M sodium veronal buffer, pH 8.6, for 25 min at 200V. The numbers at right at each panel indicate, from the top to the **bottom**, the concentration (%) values for albumin and for the globulins α 1 and α 2, β 1 and β 2, γ 1 and γ 2.

TABLE 3

Electrophoretic distribution of proteins from horses hyperimmunized with *Bothrops* venoms. Comparison with sera from non immunized horses.

Bothrops antivenoms	Horse plasma proteins (%)			
		Globulines		
	albumin	α	β	γ
(1) *B. alternatus*	33.1	16.1	16.7	34.1
(2) *B. atrox*	23.9	13.9	28.6	33.6
(3) *B. cotiara*	27.6	17.9	24.4	30.1
(4) *B. erythromelas*	45.5	15.1	15.6	23.8
(5) *B. insularis*	27.9	17.9	22.1	32.1
(6) *B. jararaca*	25.5	15.4	25.7	33.4
(7) *B. jararacussu*	27.4	14.5	15.0	43.1
(8) *B. moojeni*	21.8	16.6	16.4	45.2
(9) *B. neuwiedi*	32.1	15.3	12.3	40.3
(10) *B. pradoi*	25.1	12.9	24.9	37.1
Mixture of (2) + (5) + (7) + (8) + (9) (BUM)	20.5	14.9	30.2	34.4
Normal horse serum	34.3	18.1	27.4	20.2

ANTI-INDIRECT HEMOLYTIC ACTIVITY

The *in vitro* ability of different *Bothrops* antivenoms to neutralize the indirect hemolytic activity of *Bothrops* venoms was assayed. The following results were obtained: a) *B. cotiara* venom, at least under the conditions used in these experiments, was free of indirect hemolytic activity even when high concentrations up to 60 µg were used; b) the anti-*B. alternatus* serum at 1:100 dilutions was able to block 50% of the hemolytic activity present in the venom from all nine *Bothrops* species tested; c) the antisera against the venoms from the other *Bothrops* species have very weak or no anti-hemolytic activity; d) the anti-hemolytic activity present in the *Bothrops* polyvalent antivenom was manifested against the *B. alternatus* venom but not against the venom from the other *Bothrops* species.

Antibodies detected by the quantitative precipitin assay

Fig. 2 shows that in all *Bothrops* venom-antivenom systems analyzed with the addition of increasing quantities of venoms the amount of the corresponding antigen-antibody precipitate increases until it reaches a maximum and then declines. Inspection of the shapes of these curves shows that both the maximum quantity of precipitated antibodies and the antigen-antibody ratio vary for those representing the antivenom analyses against the individual *Bothrops* venoms or against the BVM mixture of *Bothrops* venoms used to produce antivenoms for therapeutic purposes, shows that both the maximum quantity of precipitated antibodies and the ratio antigen-antibody vary. In order to ascertain if each antivenom has comparable precipitation activity against the specific venom and against the *Bothrops* venom

mixture the corresponding curves were transformed in straight lines and the ''F test'' applied on their respective intercepts and slopes. The values were calculated for the intercepts and slopes and for the parameters used to determine ''Fa'' and ''Fb'' used to verify the parallelism and coincidence among the straight lines for each individual antivenom assayed against the specific venom and against the *Bothrops* venoms Table 4. The values for ''Fa'' and ''Fb'' being below the calculated criticals $F_{1.4} = 7.71$ and $F_{1.5} = 6.61$ respectively, indicate parallelism and coincidence of the corresponding precipitin curves experimentally obtained for each venom-antivenom assayed in parallel against the specific-venom and the BVM mixture. Accordingly, all curves are parallel with these parameters while all but the *B. cotiara* venom-antivenom system are coincidents. Table 5 shows the Ag_{mx}, the Ab_{mx} precipitated, and the ratio Ab_{mx}: Ag_{mx} for all monovalent and polyvalent IgG antibodies against *Bothrops* venoms. The data suggest that: a) the precipitation curves obtained with IgG are similar to those obtained with whole horse antisera and described many years ago Heidelberger[6], against *Clostridium tetani* or *Corynebacterium diphtheriae* toxins, since insoluble precipitates were not detected in large Ab excess; b) as expected those values vary accordingly to the venom used for immunization; c) the Ab:Ag molecular ratios can not be calculated since the venoms are complex mixtures and the molecular weight for most of their components has not yet been determined; d) the IgG antibodies produced by a mixture of *Bothrops* venoms form good amounts of Ag-Ab complexes with a high Ab:Ag ratio.

Venom neutralization. Table 6 shows these results. Three observations can be drawn from these assays: a) There was a good correlation among the results obtained when the specific and the *B. jararaca* venom where used for the monovalent antivenoms produced with *B. insularis*, *B. alternatus* and *B. atrox* venoms; b) *B. pradoi*, *B. atrox* and *B. jararacussu* seen not to be good immunogens in terms of their capacity to produce neutralizing antibodies, as compared with the other venoms; c) The horse sera anti-*B. pradoi* and anti-*B. neuwiedi* venoms were more active against *B. jararaca* venom than against the corresponding venoms used for their production.

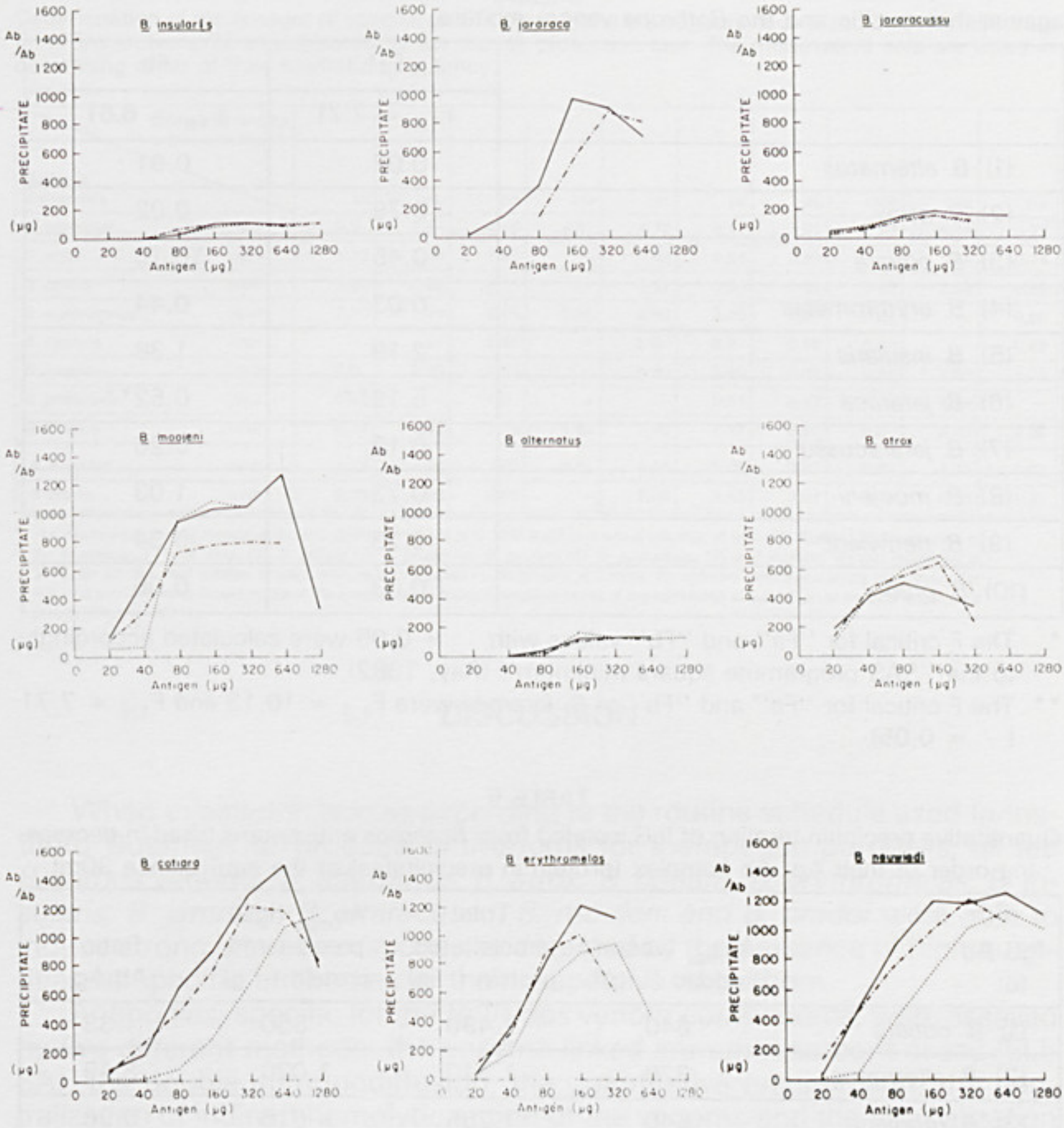

Fig. 2: Quantitative precipitin reaction between crude *Bothrops* venoms and horse antibodies to the venom componentes. 0.5ml of horse IgG dissolved in phosphate saline buffer (PBS) (1.0mg/ml) pH 7.4, was added to 0.5ml of PBS containing different amounts of the specific venom and incubated overnight at 4°C. The precipitates were washed in cold PBS, ressuspended in 0,5ml of 0.1N NaOH and the total protein determined. Control samples containing only venom or IgG were included. The ratio of antibody (Ab) to venom (Ag) found in each precipitate was plotted (axis y) against the amount of venom (axis x).
Each horse antivenom serum was assayed in parallel against the specific venom (————), against the *B. jararaca* venom (• • •), or against the mixture of *Bothrops* venoms (••••••) .

TABLE 4

Values for F critical of "Fa" and "Fb" used to verify the parallelism and the coincidence of the precipitin curves obtained for each *Bothrops* venom-antivenom system assayed both against the specific and the *Bothrops* venom mixture.

	Fa*	Fb
	$F_{1.4} = 7.71$	$F_{1.5} = 6.61$
(1) *B. alternatus*	0.07	0.91
(2) *B. atrox*	0.79	0.02
(3) *B. cotiara*	0.45	16.10
(4) *B. erythromelas*	0.03	0.44
(5) *B. insularis*	2.19	1.38
(6) *B. jararaca*	5.19**	0.52**
(7) *B. jararacussu*	0.12	0.26
(8) *B. moojeni*	0.73	1.03
(9) *B. neuwiedi*	0.23	3.34
(10) *B. pradoi*	0.03	0.06

* The F critical for "Fa" and "Fb" values with = 0.05 were calculated accordingly to the "SAS programme square minimum" (Ray, 1982)

** The F critical for "Fa" and "Fb" of *B. jararaca* were $F_{1.3} = 10.13$ and $F_{1.4} = 7.71$ (= 0.05)

TABLE 5

Quantitative precipitin titration of IgG isolated from *Bothrops* antivenoms listed in decreasing order of their Ag: Ab complex (protein in precipitates) at the equivalence 30ml.

IgG Ab to:	Ag_{max} (venom) added (g)[a]	Total precipitated protein (g)	Ab_{max} (IgG) precipitated protein (g)	Ratio Ab:Ag
(1) *B. cotiara*	640	1.490	850	1.33
(2) *B. alternatus*	320	1.340	1.020	3.19
(3) *B. moojeni*	640	1.255	615	0.96
(4) *B. neuwiedi*	640	1.243	603	0.94
(5) *B. erythromelas*	160	1.195	1.035	6.47
(6) *B. pradoi*	160	1.080	920	5.75
(7) *B. insularis*	320	1.030	710	2.22
(8) *B. jararacussu*	160	885	725	4.53
(9) *B. jararaca*	320	790	470	1.47
(10) *B. atrox*	80	505	425	5.31
(11) *Mixture of (2) + (5) + (7) + (8) + (9)* [b]	160	1.075	915	5.72

[a] Values correspond to Ag_{max} and Ab_{max} precipitated in g of total protein as determined by the Lowry's method.

[b] Solution of 50% *B. jararaca* and 50% of equal parts of the other *Bothrops* venoms designated by the numbers in parenthesis.

TABLE 6

Determination of the amount of specific and *Bothrops jararaca* venoms neutralized by 1.0ml different *Bothrops* antivenoms as estimated by the mouse protection test. The monovalent sera are listed in decreasing order of their neutralizing potency.

Neutralized venoms / antivenoms mg/ml — *Bothrops* antivenoms		alt	atr	cot	ery	ins	jar	jsu	moo	neu	pra
B. alternatus	(alt)	9,5	1,44	4,48	4,8	4,72	9,1	0,91	0,82	5,14	0,77
B. atrox	(atr)	1,15	1,09	1,87	–	1,42	0,94	0,97	–	1,54	0,74
B. cotiara	(cot)	2,20	0,83	8,1	–	3,37	0,53	0,97	0,92	2,96	0,65
B. erythromelas	(ery)	2,50	1,73	2,65	5,80	2,86	2,70	0,86	0,82	–	0,88
B. insularis	(ins)	–	–	2,65	–	8,0	8,0	0,86	0,82	–	0,88
B. jararaca	(jar)	4,0	1,20	2,55	–	6,30	3,40	0,91	0,82	2,06	1,03
B. jararacussu	(jsu)	0,84	–	1,0	–	–	0,53	0,97	–	3,28	–
B. moojeni	(moo)	0,77	1,41	2,19	1,62	1,45	2,17	0,91	4,8	3,70	2,96
B. neuwiedi	(neu)	1,78	1,20	2,65	5,6	4,86	5,05	0,91	0,82	2,81	1,02
B. pradoi	(pra)	0,85	0,83	2,66	–	1,38	3,43	0,97	1,18	4,30	1,31

* The mixture containing monospecific anti-*Bothrops* venom or 0.15M NaCl plus equal volumes of different dilutions of the specific or the *B. alternatus* (1), *B. atrox* (9), *B. cotiara* (2), *B. pradoi* (8), *B. insularis* (5), *B. jararacussu* (7) or *B. moojeni* (8) was incubated at 37°C for 30 min. Half milliliter of each mixture was injected i.p. in groups of 8 mice, the animals maintained under observation for 48h, the mortality ratio determined and the amount of neutralized venom in terms of *mg* calculated as a function of their correspondent probits (Finney, 1971).

DISCUSSION

When injected in horses according to the routine schedule used in Instituto Butantan to produce antivenoms for therapeutic purposes, all ten *Bothrops* venoms, *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni and B. pradoi* were able to induce strong immune response as detected by the presence of high quantities of specific antibodies in their respective antivenom.

Antibodies, specific for the *Bothrops* venom components, were assayed by five different methods: the enzyme-linked immunobsorbent assay (ELISA), the double immunodiffusion, the quantitative precipitation, the neutralization of indirect hemolytic action of the venoms and the neutralization of the venom toxic effects (NVTE).

The data obtained from ELISA and double immunodiffusion assays demonstrated that all *Bothrops* antivenoms contain high quantities of antibodies either reacting against antigenic components found in the venom used to immunize the animal or cross-reacting with components present in the other venoms. The discrepancies observed among the titers obtained by the ELISA and by the double immunodiffusion assays can be explained by the sensitivity limit inhibited by these two methods: for example, the ELISA assay is capable of detecting antibodies in the range of μg to ng N Ab/ml whereas in the double immunodiffusion assay this limit goes up to 5 to 10 μg N Ab/ml Bier *et al.*[1].

The high quantities of antibody in the *Bothrops* antivenoms was confirmed by the quantitative measurement of precipitins. Each antivenom was assayed by the precise analytical method introduced by Heidelberger[6], either against the specific venom, or against the *B. jararaca* venom or against the BVM. Fig. 2 shows that the precipitation curve comprises three distinct segments: an initial ascending portion, a plateau corresponding to the precipi-

tation maximum, and a descending terminal segment. The shape of these curves (floculation type) is very similar to that described for horse antisera to diphteria and tetanus toxins since they do not appear to go through the origin (Heidelberger, 1949). The presence of a soluble antigen and antibody complex at the antibody excess region as well as in the antigen excess region explaing this kind of curve. The mechanism acounting for the floculation type of curve may be related with the floculation antibody heterogeneity and with the relative binding affinities of these antibodies as compared to the precipitin antibodies. Inspection of the three floculation curves obtained for each *Bothrops* antivenom indicates that they follow similar patterns. There are, therefore, indications suggesting that the antibody population found in each antivenom recognizes similar antigens found either in the specific venom or in the *B. jararaca* one as well as in the BVM mixture. To assert this suspicion the floculation curves obtained for each horse monovalent *Bothrops* antivenom assayed both against the corresponding venom and the BVM were transformed in straight lines and their intercepts and slopes were submitted to F test. With the aid of the parameters used to calculate the values for ''Fa'' and for ''Fb'' and their corresponding ''critical F'' (Table 4), it was possible to conclude that all paired curves were parallel and coincident. The *B. cotiara* antivenom pair was an exception for although being parallel it was not coincident.

With the exception of *B. atrox* antivenom the amount of antigen antibody complexes found at the zone of equivalence with the other nine monovalent antivenoms was over 1,0 mg/ml of the total protein content. These values were comparable to those obtained with the polyvalent antivenom (Table 5).

The immunochemical assays indicate therefore that each *Bothrops* venom per se was able to induce the production of antibodies capable of mutually cross-react against one each other. The presence of such complex antibody populations was reflected on their ability to neutralize the biological properties of the venoms. For instance, the monovalent antivenoms with higher number of precipitating properties detected by quantitative measurement of antigen-antibody interactions (anti-*B. alternatus*, anti-*B. cotiara*, anti-*B. insularis*, anti-*B. erythromelas* and anti-*B. moojeni*) possess also strong neutralizing activity against the venom lethal effects. On the other hand, the *B. jararacussu* antivenom contains both small amounts of precipitating antibodies and low neutralizing activity (Table 5 and 6). However, such correlation was not found when the anti-indirect hemolytic activity was assayed Lachmann *et al.*, Gutierrez *et al.*,[8,5]; such activity was almost exclusive of the *B. alternatus* antivenom.

The increase of the γ -globulins at the end of the immunization procedure is compatible with the high quantities of antibodies detected in the *Bothrops* antivenoms.

In conclusion: a) all ten *Bothrops* venoms were able to induce antibody formation wich mutually cross-react as assayed by three different immunochemical methods; b) the venoms from *B. alternatus, B. jararaca, B. moojeni* and *B. neuwiedi*, four of the most spread species of *Bothrops* snakes in Brazil Hoge *et al,*[7], behaved as good immunogens for horses and the antivenoms elicited, although in different degree, are capable to neutralize the lethal activity present in the other venoms; c) in contrast, *B. jararacussu* venom behaved as poor immunogen and its lethal activity was not efficiently

blocked neither by the specific antivenom nor the antivenoms produced by the other *Bothrops* venoms. These results agree well with previous work showing that venoms from six *Bothrops* species contain common antigenic components (Villarroel et. al[16]; Villarroel et al[17]). Taken together the observations described in this work indicate that the *Bothrops* venom mixture to be used to immunize horses to produce antivenom for therapeutic purposes should contain venoms from *B. alternatus, B. jararaca, B. jararacussu, B. moojeni* and *B.neuwiedi. Table 6 shows that the antivenoms elicited by these five Bothrops* venoms contain antibodies capable to neutralize the lethal activities for mice present in all ten *Bothrops* venoms used in this work.

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RESUMO: Cavalos foram imunizados com veneno de *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, B. newiedi,* e *B. pradoi*. Anticorpos específicos para componentes antigênicos dos venenos foram detectados pelos métodos imunoenzimático, dupla-difusão e precipitação quantitativa enquanto que os anticorpos neutralizantes foram analisados pelos métodos da hemólise indireta em placas e pela neutralização de seus efeitos letais. Anticorpos, tanto específicos como dando reações cruzadas com venenos botrópicos foram encontrados em todos os dez soros monovalentes. Modificações nos padrões eletroforéticos, caracterizadas por uma redução no pico da albumina e por um correspondente aumento das γ -globulinas com modificações ora acentuadas ora pouco perceptíveis nas frações das α e β globulinas, foram detectadas em todos esses soros.
UNITERMOS: Antivenenos; antissoros botrópicos monovalentes; venenos botrópicos.

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VARIAÇÃO DO COMPORTAMENTO BIOLÓGICO DO TUMOR HUMANO KB, HETEROTRANSPLANTADO SERIADAMENTE EM RATOS ''NUDE'' ATÍMICOS*

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RESUMO: Foram analisados, estatisticamente, os resultados obtidos de estudo de 18 passagens seriadas do tumor humano KB transplantado em ratos nude atímicos. Foram abordados os aspectos de crescimento ou regressão da massa tumoral, bem como a caquexia por vezes detectada no receptor.

UNITERMOS: Rato nude atímico. Tumor humano KB. Hetero-transplante.

INTRODUÇÃO

Em animais ''nude'' atímicos, a pega inicial de heterotransplantes de tumores humanos é mais facilmente obtida a partir de linhagens de culturas celulares do que quando os transplantes são realizados com tecidos frescos oriundos de biópsias [4,5,6,8,9]. Por outro lado, a baixa transplantabilidade continuada desses heterotransplantes tem dificultado estudos sobre a variação do comportamento biológico dessas neoplasias transplantadas, bem como a análise dos mecanismos imunológicos de defesa desses animais frente ao tumor.

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No presente trabalho, apresentamos os resultados obtidos em uma série de 18 passagens de heterotransplantes de tumor humano KB (carcinoma epidermoide de boca) em ratos ''nude'' atímicos, realizados no período de 1985 a 1987, analisando-se principalmente o aspecto do desenvolvimento da massa tumoral, seja no crescimento ou regressão, com algumas considerações sobre a caquexia por vezes detectada no receptor.

Nosso objetivo principal foi o de avaliar, através da análise estatística dos resultados, o grau de associação existente entre as variáveis estudadas e a malignidade do tumor no decorrer das passagens seriadas.

MATERIAIS E MÉTODOS

Animais: Foram utilizados, para os heterotransplantes, 134 ratos nude atímicos adultos (⩾ 1 mês de idade), de ambos os sexos. Esses animais foram mantidos antes e durante a fase de experimentação em condições especiais de biotério, em sala equipada com unidade de ventilação estéril e temperatura e umidade do ar aproximadamente constantes. Alimento e água eram previamente esterilizados e oferecidos ''ad libitum''.

Tumor KB: Os animais foram heterotransplantados com tumor humano KB, uma linhagem de carcinoma epidermoide de boca humano, mantido em cultura desde 1955[7].

Inoculações: A primeira inoculação, designada como passagem zero (P0), foi realizada a partir de tripsinização da cultura e ajuste da suspensão celular para 1 x 10^6células/ml, tendo sido inoculado um volume final de 1 ml/animal. As demais inoculações foram realizadas por passagem seriada de fragmentos de cerca de 1 mm^3 do tumor de um animal a outro, com auxílio de trocater, após excisão da massa em crescimento, em condições de assepsia e fragmentação da mesma em meio Eagle. Tais passagens seriadas foram codificadas de P1 a P18 e agrupadas para fins de análise estatística, de modo a conter aproximadamente o mesmo número de animais.

Todas as inoculações foram realizadas subcutaneamente na região axilar esquerda dos animais.

As seguintes variáveis foram analisadas no desenvolvimento do tumor transplantado.

1. **Crescimento de massa tumoral**

A primeira visualização objetiva de um abaulamento na região inoculada é tida como o início de crescimento do tumor transplantado. A incidência assim considerada dessa pega foi de 78%. O tempo decorrido (em dias) desde a inoculação até a visualização do abaulamento variou de 6 a 46 dias.

Quando a massa tumoral atinge um tamanho que dificulta a própria locomoção do animal (cerca de 4,5cm no seu maior eixo) (Foto 1), este é sacrificado e sua massa é extirpada e retransplantada em outros animais.

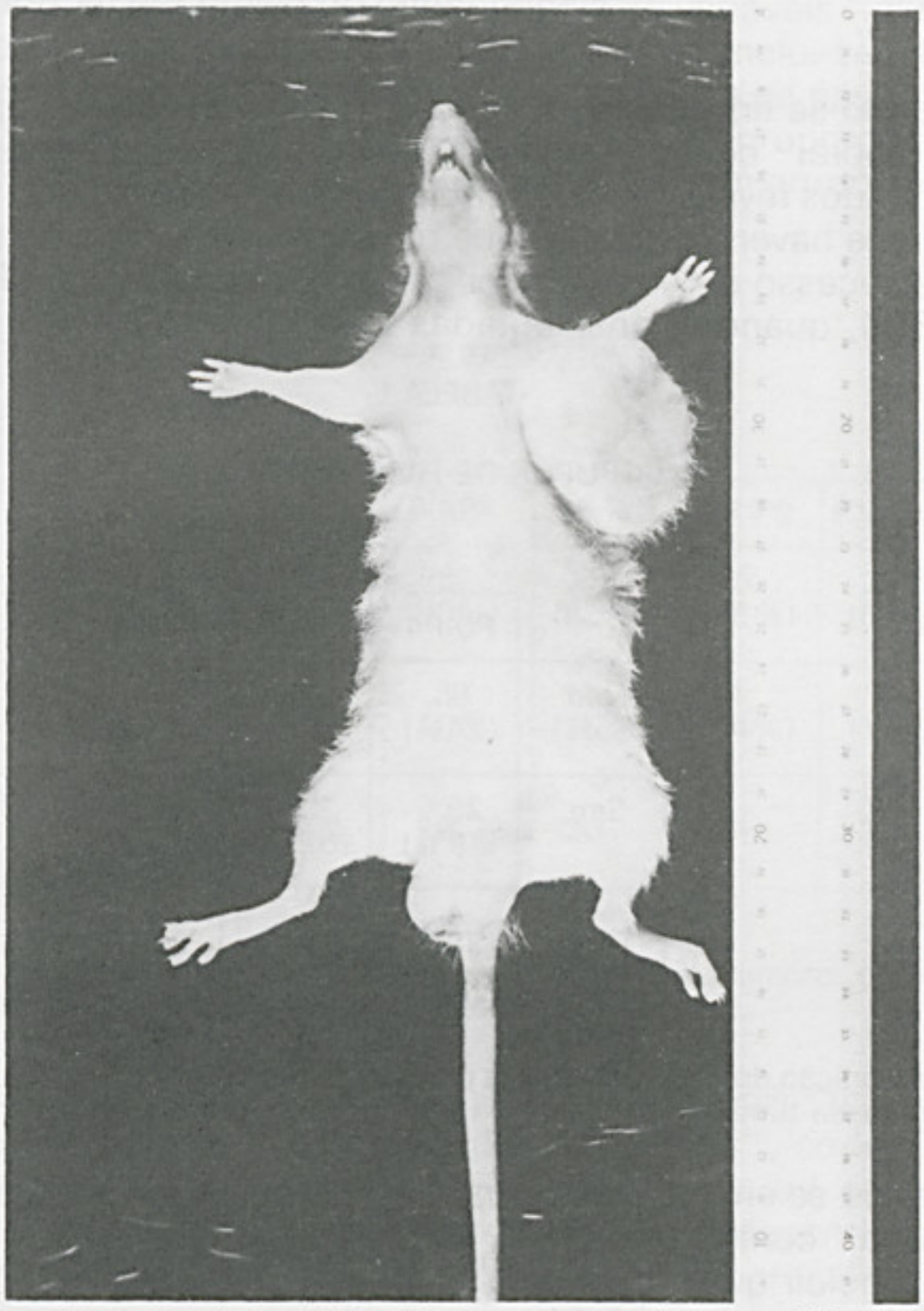

Foto 1: Exemplar de rato nude portador de massa tumoral (tumor KB), desenvolvida na região de inoculação do transplante.

2. **Regressão**:

Em cerca de 12% dos casos, após o crescimento constatado pelo abaulamento, atingindo em média 4,0cm no seu diâmetro maior, o tumor sofreu processo de regressão, culminando com o desaparecimento total da massa anteriormente detectada. O tempo (em dias) para a ocorrência de tal fenômeno, considerado desde o aparecimento da massa até o início de seu desaparecimento, variou de 21 a 78 dias.

3. **Caquexia**:

Trinta e quatro dos animais inoculados (25%) entraram em estado de caquexia e foram então sacrificados para análises complementares. Desses animais, 19 haviam apresentado massa e 15 não. O tempo decorrido para atingir esse estado variou de 22 a 108 dias, a contar do dia em que foram inoculados.

As variáveis acima descritas foram analisadas estatisticamente por testes de homogeneidade (qui-quadrado), em tabelas de contingência e testes para o risco relativo.

RESULTADOS

Associando-se em tabela de contingência as variáveis ''aparecimento de massa tumoral'' contra ''sucessão de passagens'', a análise estatística desses resultados revela que há uma dependência entre esses dois fatores, ou seja, parece haver indícios de que passagens mais iniciais (P0-P6) tiveram menos sucesso de pega que passagens intermediárias (P7-P9) ou finais (P10-P18), quando transplantadas (Tabela I).

TABELA I

		GRUPOS DE PASSAGENS				
		P0-P4	P5-P6	P7-P9	P10-P18	Total
Aparecimento da massa	Não	9 (24%)	12 (38%)	4 (14%)	4 (14%)	29 (22%)
	Sim	29 (76%)	20 (62%)	24 (86%)	32 (89%)	105 (78%)
	Total	**38**	**32**	**28**	**26**	**134**

p = 0.04

Tabela I: Distribuição dos ratos conforme o grupo de passagem e o aparecimento ou não da massa tumoral.

Associando-se em tabela de contingência as variáveis ''tempo para aparecer a massa'' contra ''sucessão de passagens'', as estimativas obtidas permitem concluir que o tempo para aparecer a massa tumoral aumenta nos grupos de passagens P5-P9, em relação ao grupo de passagem P0-P4, e diminui no grupo P10-P18 em relação aos grupos P5-P9 (Tabela II).

TABELA II

		GRUPOS DE PASSAGENS				
		P0-P4	P5-P6	P7-P9	P10-P18	Total
Tempo p/aparecer a massa (em dias)	6-8	10 (34%)	3 (15%)	3 (13%)	11 (34%)	27
	9-12	8 (28%)	4 (20%)	7 (29%)	16 (50%)	35
	13-17	8 (28%)	8 (40%)	9 (38%)	5 (16%)	30
	Total	**29**	**20**	**24**	**32**	**105**

p = 0.024

Tabela II: Distribuição dos ratos conforme o grupo de passagem e o tempo para aparecer a massa.

Associando-se em tabela de contingência as variáveis ''aparecimento da regressão'' contra ''sucessão de passagens'', conclui-se não haver indícios de que a regressão da massa tumoral dependa da passagem utilizada no transplante (Tabela III). Note-se que a proporção de ''não aparecimento de regressão'' varia muito pouco de um grupo de passagem para outro.

TABELA III

GRUPOS DE PASSAGENS

		P0-P4	P5-P6	P7-P9	P10-P18	Total
Aparecimento da regressão	Não	26 (90%)	17 (85%)	23 (96%)	26 (81%)	92 (88%)
	Sim	3 (10%)	3 (15%)	1 (4%)	6 (19%)	13 (12%)
	Total	**29**	**20**	**24**	**32**	**105**

p = 0.45

Tabela III: Distribuição dos ratos que desenvolveram crescimento tumoral, conforme o grupo de passagem e a ocorrência ou não de regressão.

Associando-se em tabela de contingência as variáveis ''tempo para aparecer a regressão'' contra ''sucessão das passagens'', conclui-se que há indícios de que há dependência direta entre esses fatores, embora não tenha sido possível detectar como se comporta essa dependência, devido ao número insuficiente de ratos que apresentaram regressão (Tabela IV).

TABELA IV

GRUPOS DE PASSAGENS

		P0-P4	P5-P6	P7-P9	P10-P18	Total
Tempo p/aparecer a regressão (em dias)	21-55	0	3	0	4	7
	56-78	3	0	1	2	6
	Total	**3**	**3**	**1**	**6**	**13**

p = 0.07

Tabela IV: Distribuição dos ratos conforme o grupo de passagem e o tempo para aparecer a regressão.

O teste de homogeneidade em tabela de contingência entre as variáveis ''aparecimento da regressão'' e ''tempo para aparecer a massa'' não mostrou indícios de dependência entre essas duas variáveis (Tabela V).

TABELA V

TEMPO P/ APARECER A MASSA (EM DIAS)

		0-8	9-12	13-17	18-47	Total
Aparecimento	Não	21	30	28	13	92
da regressão	Sim	6	5	2	0	13
	Total	**27**	**35**	**30**	**13**	**105**

p = 0.40

Tabela V: Distribuição dos ratos conforme o tempo para aparecer a massa e o aparecimento ou não da regressão.

Da mesma forma, quando as variáveis "tempo para aparecer a massa" e "tempo para aparecer a regressão" são confrontadas num diagrama de dispersão (Fig. 1), conclui-se não haver indícios de que o tempo para aparecer a massa dependa linearmente do tempo para aparecer a regressão (correlação = — 0,063).

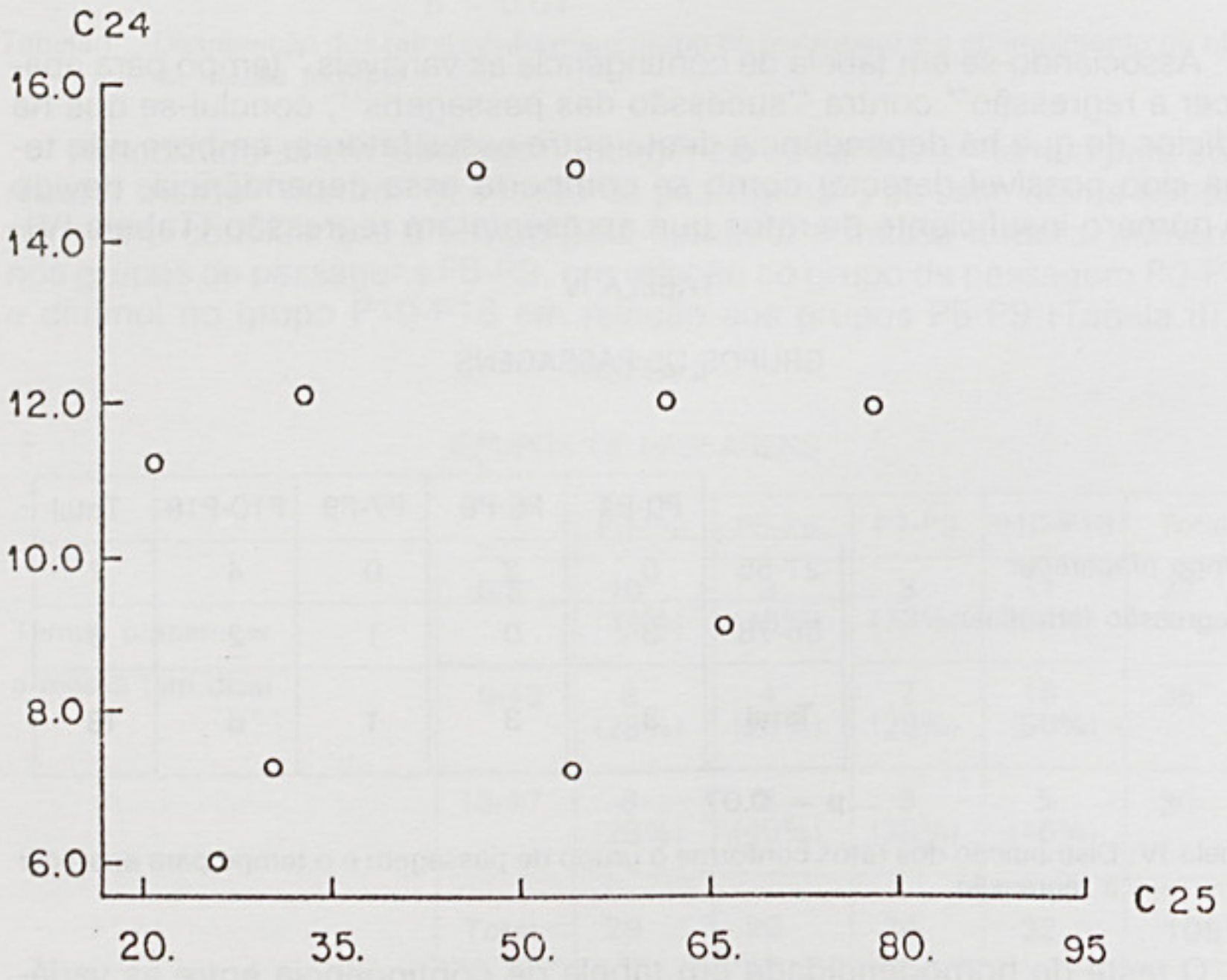

Figura 1: Diagrama de dispersão entre as variáveis "tempo para aparecer a massa" (C 24) e "tempo para aparecer a regressão" (C 25).

Com relação ao fator "caquexia", aplicando-se o teste não paramétrico para o risco relativo[1], conclui-se que não há indícios de que a passagem em que o tumor foi inoculado, ou que o tempo para aparecer a massa tumoral, influenciem no aparecimento da caquexia.

DISCUSSÃO E CONCLUSÕES

O valor de um modelo biológico experimental depende de sua estabilidade, seja concernente às semelhanças existentes com o modelo original, seja concernente à sua manutenção e repetitividade a longo termo.

No modelo rato nude atímico transplantado com tumor humano a estabilidade consiste na permanência da identidade original do tumor humano e a repetitividade consiste em passar esse tumor no animal por período de tempo suficiente que permita investigações biológicas do transplante e do transplantado, ou mesmo estudos básicos terapêuticos.

Wallace et al, 71[11] estudaram o crescimento de células KB em hamsters recém-nascidos tratados com soro anti-timocítico anti-hamster (ATS) ou soro normal de coelho (NRS). Uma única injeção de ATS pode promover a formação de tumor KB metastático numa maior proporção de animais, enquanto que apenas poucos animais dos tratados com NRS desenvolviam pequenos tumores que ocasionalmente regrediam.

Sudo, 87[10] também relatou o transplante dessa mesma linhagem KB em camundongos nude, porém esplenectomizados e irradiados.

Nosso modelo experimental rato nude atímico/tumor humano KB sem nenhum outro artefato, vem sendo mantido em passagens seriadas em nosso laboratório desde 1985[3], encontrando-se atualmente na passagem 29.

Este nosso estudo, concernente à estabilidade do modelo a longo termo, compara bioestatisticamente as diferenças observadas entre as passagens iniciais do tumor e as passagens mais avançadas.

Assim, essa análise indica que, à medida que o número da passagem aumenta, há uma maior probabilidade de aparecer a massa tumoral no animal transplantado, mostrando haver uma interação entre a estrutura antigênica da célula tumoral e a competência do microambiente do hospedeiro.

Corroborando ainda essa interação transplante/hospedeiro, pudemos verificar que em passagens intermediárias e finais o tempo requerido para o aparecimento do crescimento tumoral foi menor que nas passagens iniciais.

As regressões tumorais observadas (12%) podem ser explicadas pelos mecanismos de defesa individuais do hospedeiro, atuando contra o crescimento tumoral. Essa regressão não se mostrou dependente da passagem mais ou menos avançada do tumor, nem do tempo requerido para o aparecimento do crescimento tumoral, nem ainda há indícios de que o tempo para aparecer a massa tumoral dependa do tempo para aparecer a regressão.

No que tange o fator caquexia, presente na maioria dos ratos inoculados, após um período variável de 22 a 108 dias, não há indícios de que a passagem em que o tumor foi inoculado influencie sobre esse fator e nem há indícios de que o tempo levado para aparecer a massa tumoral influencie no aparecimento do estado de caquexia.

Os resultados no caso do crescimento da massa tumoral, vieram cor-

roborar achados histológicos que sugerem o aumento da malignidade do tumor seriadamente transplantado[2].

Assim, à medida que as passagens seriadas se sucederam, os valores médios do volume cariométrico das células componentes do tumor, aumentaram significantemente e, da mesma forma, o número de mitoses aumentou consideravelmente.

ABSTRACT: The results obtained from the study of 18 serial passages of human tumor KB transplanted in athymic nude rats have been statistically analysed. Aspects of growth or regression of the tumoral mass, as well as the cachexy sometimes detected in the receptor, have been approached.

KEY WORDS: Athymic nude rat. Human tumor KB. Heterotransplant.

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BIOCHEMICAL STUDIES OF THE SEA SNAKE NEUROTOXINS

Anthony T. TU
Roger A. MILLER

ABSTRACT: Sea snakes belonging to the family Hydrophiidae are marine-adapted, serpents found widespread in tropical and subtropical costal waters of the Indian and Pacific Oceans. Sea snake venom is a mixture of different proteins and contains potent neurotoxins. The LD_{50} for sea snake venom can be as low as 0.01 mg/Kg. The purified type I postsynaptic neurotoxins consist of 60-62 amino acid residues with four disulfide bonds. They are basic proteins with isoelectric points of 9 to 10 and range in molecular weight from 6,000 to 8,000. Sea snake neurotoxin work in this laboratory has centered on the postsynaptic neurotoxin of *Laticauda semifasciata, Lapemis hardwickii, Pelamis platurus* and *Acalyptophis peronii*. Sea snake neurotoxins show considerable homogeneity in their amino acid sequences with many invariant residues. Raman studies indicate the neurotoxins are a mixture of beta sheet and beta turns with no alpha helical secondary structure. The origin of lethality comes from the fact the sea snake neurotoxin strongly binds to the acetylcholine receptor at the neuromuscular junction which leads to muscle paralysis and respiratory arrest. Chemical modification of the conserved tryptophan residue has lead to the loss of the specific binding of the acetylcholine receptor and the loss of toxicity, but the modified toxin retained the ability to bind to neurotoxin antibodies. This suggested that neurotoxins can be converted into toxoids. A single tyrosine residue, some arginine and lysine residues are also essential to neurotoxicity. In addition to specific residues, some regions of polypeptide backbone are also important for toxicity.
KEY WORDS: Neurotoxins, Venoms, Acetylcholine Receptor.

INTRODUCTION

The scope of this review and discussion is restricted mainly to the work in this laboratory related to sea snake neurotoxins and interaction with acetylcholine receptors (AChR) in order to restrict the length of the review. There

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are many review articles on this subject, and readers are encouraged to read these for an overall view of neurotoxins.[1-4]

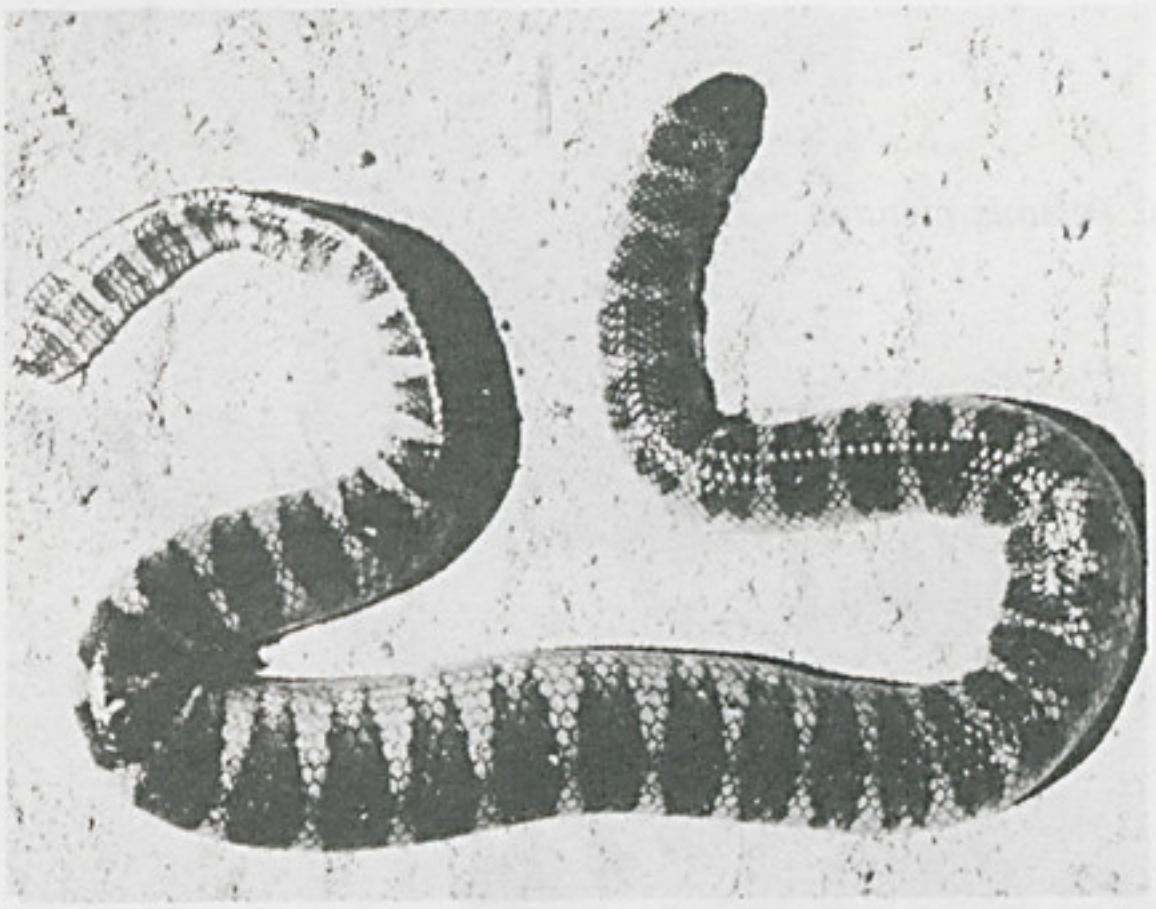
The sea snake is a marine-adapted serpent belonging to the family of Hydrophiidae and they are found widespread in tropical and subtropical coastal waters of he Indian and Pacific Oceans, however they are not found in the Atlantic Ocean. (Fig. 1) There are many varieties of sea snakes with different colors, shapes, and sizes. They are well adapted for the marine environment and have a flat tail and a salt gland. There are two subfamilies within the family Hydrophiidae. They are Hydrophiinae and Laticaudinae. The two types of sea snakes have distinct differences in their ventral scales. The former usually does not have ventral scales distinguishable from the surrounding scales, whereas the latter has wide ventral scales. These differences in ventral scale patterns eventually dictate their habitats. The reason a snake can crawl is due to the movement of its ventral scales. Since Hydrophiinae do not have ventral scales, they spend their entire lives in the sea. On the other hand, the Laticaudinae can swin in the sea and crawl on the beach and rocks.

Fig. 1. Distribution of sea snakes

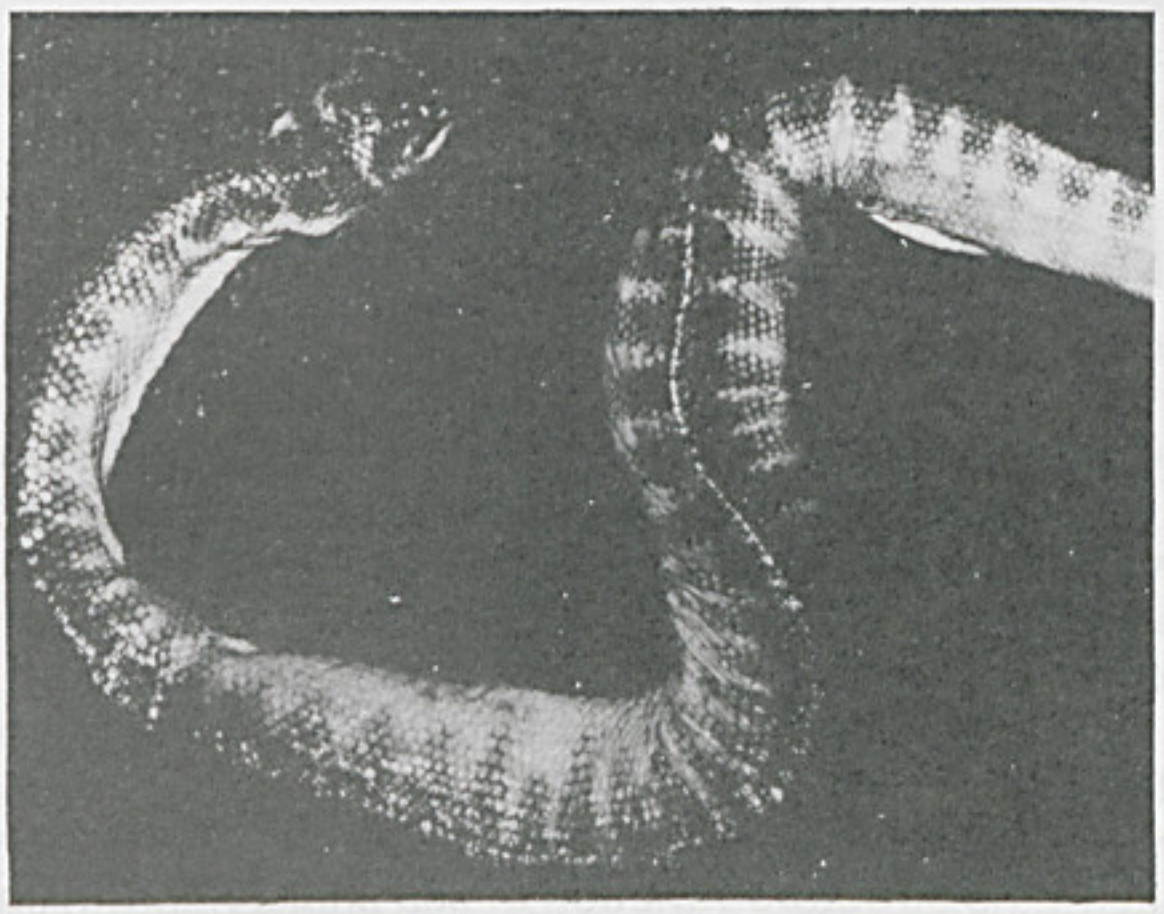
Sea snake venom is a mixture of different proteins, of which some are toxic and some components are relatively nontoxic. Because a venom contains highly toxic postsynaptic neurotoxins venom as a whole exhibits high toxicity. The potent neurotoxins in their venoms can cause muscle paralysis and respiratory failure of a victim which may lead to morbidity or death.

Most of the sea snake work in this laboratory has centered on the venom from four species of sea snakes: *Laticauda semifasciata* captured in the waters near the Philippines (Fig. 2A), *Lapemis hardwickii* captured in the Gulf of Thailand (Fig.2B), *Pelamis platurus* captured in the Pacific coastal waters of Costa Rica (Fig. 2C) and *Acalyptophis peronii* captured in the Gulf of Thailand (Fig. 2D).

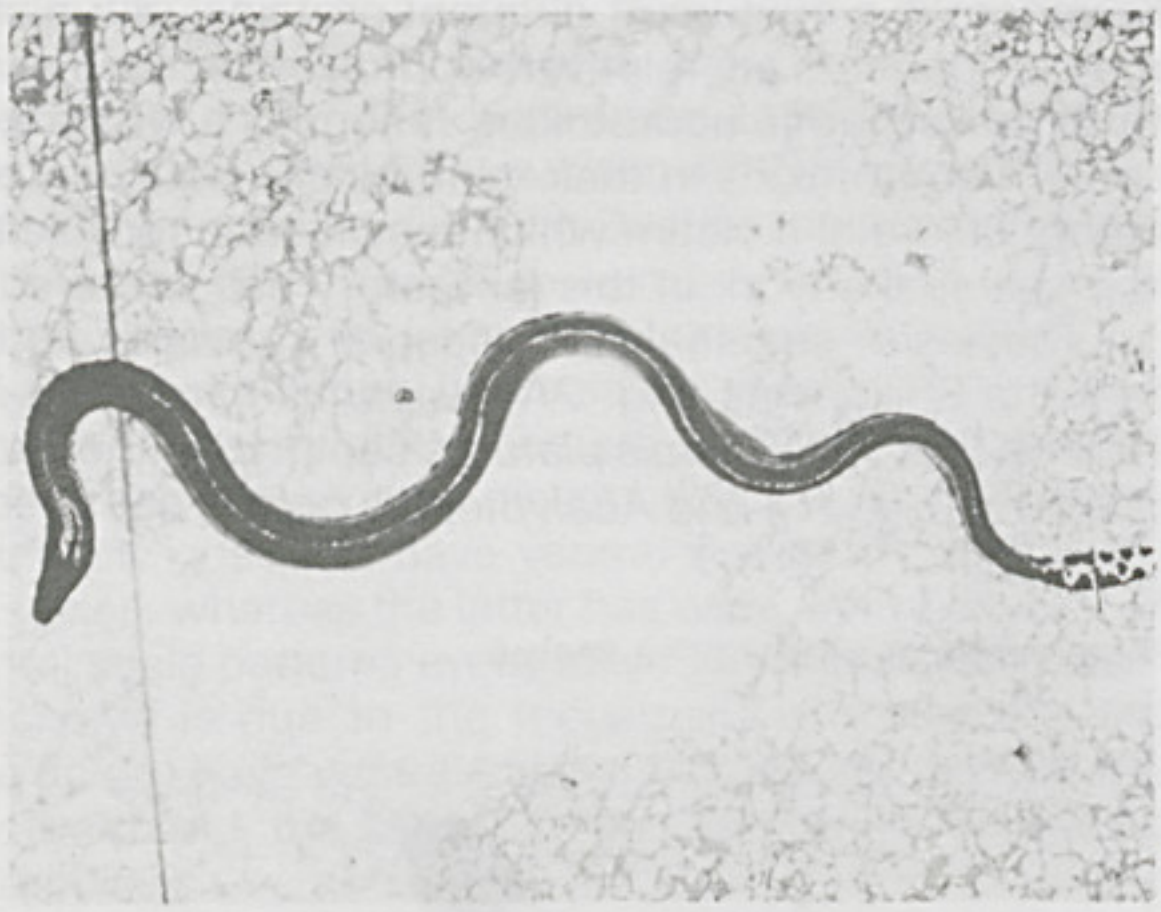
Fig. 2. (A-D) Photographs of selected sea snakes



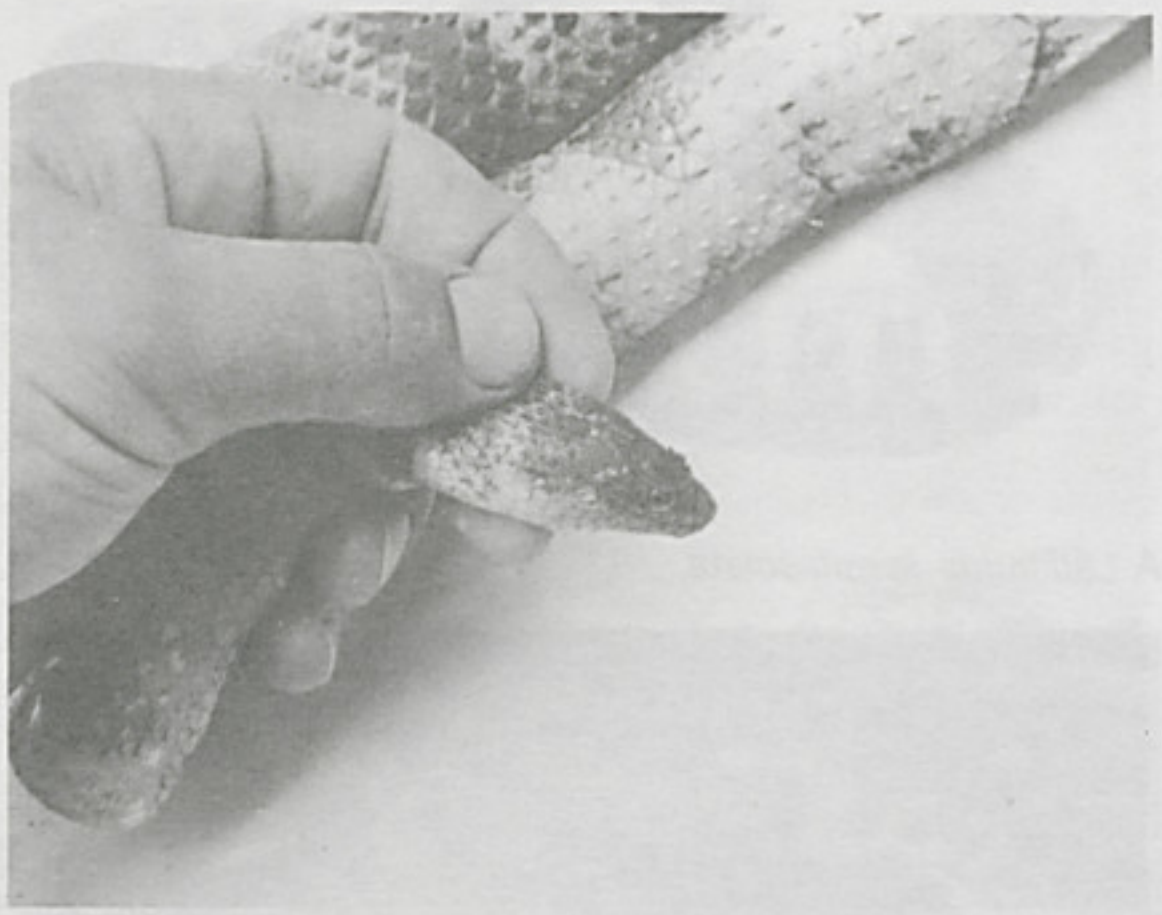
A *Laticauda semifasciata*



B *Lapemis hardwickii*



C *Pelamis platurus*



D *Acalyptophis peronii*

The yellow-bellied sea snake, *Pelamis platurus*, which is considered to be the most widespread sea snake in the world, is found in most of the coastal waters shown in Figure 1.[5] *Acalyptophis peronii* is the third most common sea snake in the Gulf of Thailand. The appearance, color pattern, and other morphological characteristics of this snake are quite different from other sea snakes. This sea snake is readily recognizable by its horn-like lifted-up scale near the eye.[1,6,7]

The remainder of this review will summarize the work in this laboratory in understanding the structure-function of sea snake neurotoxins and interaction studies with the AChR.

STRUCTURE FUNCTION RELATIONSHIPS

All sea snakes are poisonous and their venoms are extremely toxic. The LD_{50} for crude sea snake venom can be as low as 0.01 mg/Kg mouse body weight. For purified neurotoxin the LD_{50} is even lower, suggesting the high toxicity of sea snake venoms.[1]

Although the toxicity of sea snake venom is high, the yield of venom from a sea snake is very small with yields of 0.6 to 19.0 mg per snake, depending on the species.[8,9]

When various sea snake venoms were examined using immunodiffusion methods different venoms immunologically crossinteracted with each other (Fig. 3) indicating the close similarity in the composition of the venoms among different sea snakes.[10,11]

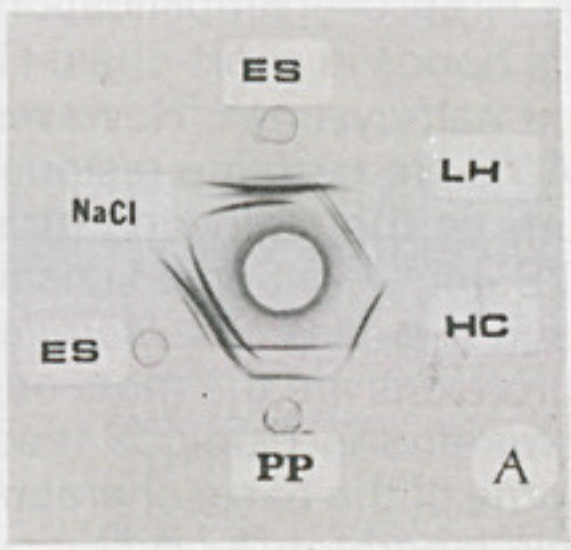


Fig. 3 Immunodiffusion study of sea snakes venoms
Immunologic cross-reaction of different sea snake venoms.
Antibody used was from anti-*E. schistosa* venom manufactured by Commonwealth Serum Laboratory, Melbourne, Australia. ES, *E. shistosa;* LH, *L. hardwickii;* HC, *H. cyanocinctus;* PP, *P. platurus.*

Sea snake venom seems to lack cytolytic activity. When venoms of various sea snakes *(E. schistosa, L. semifasciata, L. hardwickii, and P. platurus)* were added to the cell cultures of KB cells, Yoshida sarcoma cells, and normal peritoneal cells, no lysis was observed.[12]

Most sea snake neurotoxins consist of 60-62 amino acid residues with four disulfide bonds. These are the type I or short-chain neurotoxins. However, several type II or long-chain toxins were also isolated. Both types I and II are postsynaptic toxins, but type II toxins have five disulfide bonds. Some neurotoxins have structures between type I and II; they contain four disulfide bonds but have many other features similar to type II neurotoxins.[1]

Most sea snake venoms seem to contain only the postsynaptic neurotoxin. Only in *Enhydrina schistosa* venom, which also possesses a postsynaptic toxin, was a presynaptic type found and identified as phospholipase A. Therefore, to classify neurotoxins solely on the basis of their disulfide bonds or amino acid sequences is an oversimplification.[1]

There are four disulfide bonds in short-chain (Type I) neurotoxins which means that there are eight half-cystines. However, all Hydrophiinae toxins have nine half-cystines. An extra cysteine residue can be readily detected from the Raman spectrum as the sulfhydryl group shows a distinct S-H stretching vibration at 2578 cm^{-1}.[13,14] Some Laticaudinae toxins do not have a free cysteine residue as in the case of *L. semifasciata* toxins. These two types of postsynaptic neurotoxins are the most commonly found neurotoxins in Hydrophiidae venoms and in Elapidae (cobras and kraits) venoms.

Table 1 summarizes some of the basic characteristics of selected toxins purified and studied in this laboratory.[6,7,14-16]

TABLE 1

BIOCHEMICAL CHARACTERISTICS OF SELECTED POSTSYNAPTIC NEUROTOXINS DONE IN THIS LABORATORY

Neurotoxin	MW	pI	LD_{50}	#res/molec
Laticauda semifasciata				
Toxin **b**	6,800	> 9.5	0.05 mg/Kg	62
Lapemis hardwickii				
Lapemis Toxin	6,800	9.6	0.01 mg/Kg	60
Pelamis platurus				
Toxin **b**	6,800	8.7	0.185 mg/Kg	60
Acalyptophis peronii				
Major Toxin	6,600	> 9.5	0.125 mg/Kg	60
Acalyptophis peronii				
Minor Toxin	6,600	> 9.5	0.10 mg /Kg	60

*(i.v. mice)

The amino acid sequences of many sea snake neurotoxins have been determined. The complete amino acid sequence of the neurotoxins sequenced in this laboratory are listed in Table 2.[6,7,16-18] There are two toxic fractions in the *Acalyptophis peronii* venom, the most toxic and abundant fraction was isolated and termed major toxin. The *A. peronii* minor toxin was identified and compared to that of the major toxin. The only difference between the major and the minor toxins is in the 43rd residue. The major toxin at this position contains glutamine, while the minor toxin contains glutamic acid.[6,7]

TABLE 2

AMINO ACID SEQUENCES OF SEA SNAKE POSTSYNAPTIC NEUROTOXINS COMPLETED IN THIS LABORATORY

Laticauda semifasciata
Toxin b
RICFNQHSSQPQTTKTCPSGQSSCYHKQWSDFRGTIIERGCGCPTVKPGIKLSCCESERCNN
Lapemis hardwickii
Lapemis Toxin
MTCCNQQSSQPKTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKPGIKLECCHTNECNN
Pelamis platurus
Toxin b
MTCCNQQSSEPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKSGIKLECCHTNECNN
Acalyptophis peronii
Major Toxin
MTCCNQQSSQPKTTTNCAGNSCYKKTWSDHRGTIIERGCGCPQVKSGIKLECCHTNECNN
Acalyptophis peronii
Minor Toxin
MTCCNQQSSQPKTTTNCAGNSCYKKTWSDHRGTIIERGCGCPEVKSGIKLECCHTNECNN

In order to understand the exact mechanism of the neurotoxic action, it is important to know the secondary structure of the neurotoxins as well. It is now known that postsynaptic neurotoxins attach to the a-subunits of acetylcholine receptor (AChR).[1] The conformation of sea snake neurotoxins has been extensively studied. The results of these studies are summarized in Table 3.[5,16,18,19]

Raman spectroscopic examination of pelamis toxin b indicates that the toxin contains a considerable amount of antiparallel β-structure, β-turn, and random coil without α-helix as the amide I band appears at 1673cm^{-1} and the amide III band at 1246 cm^{-1}. Circular dichroic studies also indicate a typical β sheet structure. The pelamis toxins b is a typical postsynaptic neurotoxin as it binds to the AChR competitively with a well known toxin, α-bungarotoxin.[16]

TABLE 3

CONFORMATION OF SEA SNAKE NEUROTOXINS AS DETERMINED BY RAMAN SPECTROSCOPY IN THIS LABORATORY

Venom	Toxin	Conformation
Enhydrina schistosa	Major toxin	Mixture of β turn and β sheet and no α helix
Lapemis hardwickii	Lapemis toxin	Mixture of β turn and β sheet and no α helix
Pelamis platurus	Pelamis toxin **a**	Mixture of β turn and β sheet and no α helix
Pelamis platurus	Pelamis toxin **b**	Mixture of β turn and β sheet and no α helix

TABLE 4

CHEMICAL MODIFICATION OF SEA SNAKE NEUROTOXINS IN THIS LABORATORY

Residue	Toxin: Conclusion
Arginine	
	L. semifasciata
	toxin a: No loss of toxicity when 1 of 3 residues modified
	toxin b: No loss of toxicity when 1 of 2 residues modified
Lysine	
	L. semifasciata
	toxin a: No loss of toxicity when 3 of 4 residues modified
	toxin b: No loss of toxicity when 4 of 5 residues modified
Tryptophan	
	E. schistosa
	Major toxin: Loss of toxicity
	L. hardwickii
	Lapemis toxin: Loss of toxicitv
	L. semifasciata
	Toxins a & b: Loss of toxicity
Tyrosine	
	L. hardwickii
	Lapemis toxin: Loss of toxicity
Sulfhydryl group	
	P. platurus
	Pelamis toxin: Less toxic but retains toxicity, still bind to AChR
Disulfide bond	
	P. platurus
	Pelamis toxin: Loss of toxicity

The amide I band and III band for *Enhydrina schistosa* toxin were at 1672 cm^{-1} and 1242 cm^{-1}, respectively. These wave numbers are characteristic for anti-parallel β sheet structure. The presence of β-sheet structure found by Raman spectroscopic study was later confirmed by X-ray diffraction study on *Laticauda semifasciata* toxin b.[19]

Sea snake short-chain toxins have a molecular weight of only 6,800. The small size with four disulfide bonds makes these toxins very compact and stable molecules. Therefore, when the *Pelamis platurus* toxin is subjected to heat treatment at 100 °C and subsequent cooling, it does not change its conformation substantially. Amide I and III bands and S-S stretching vibration did not change by heat treatment.[5]

The four disulfide bonds of the neurotoxin are clustered in one area and there is a protruding loop. It is suspected that this loop is the one that plays an important role in binding to the AChR. The four disulfide bonds are believed to be important for maintaining the specific conformation and have been studied extensively. The conformation of the disulfide bond in C-C-S-S-C-C network is *gauche-gauche-gauche* conformation at the S-S stretching vibration appearing at 510-512 cm^{-1} of Raman spectrum.[1,21]

Figure 4 shows the two-dimensional structure and tertiary structure of *Lapemis hardwickii* lapemis toxin adapted from the more well studied toxin b of *Laticauda semifasciata* venom.[22] Note the three major structural loops of the tertiary structure termed loop A, B, and C from the amino terminal to the carboxyl terminal respectively.

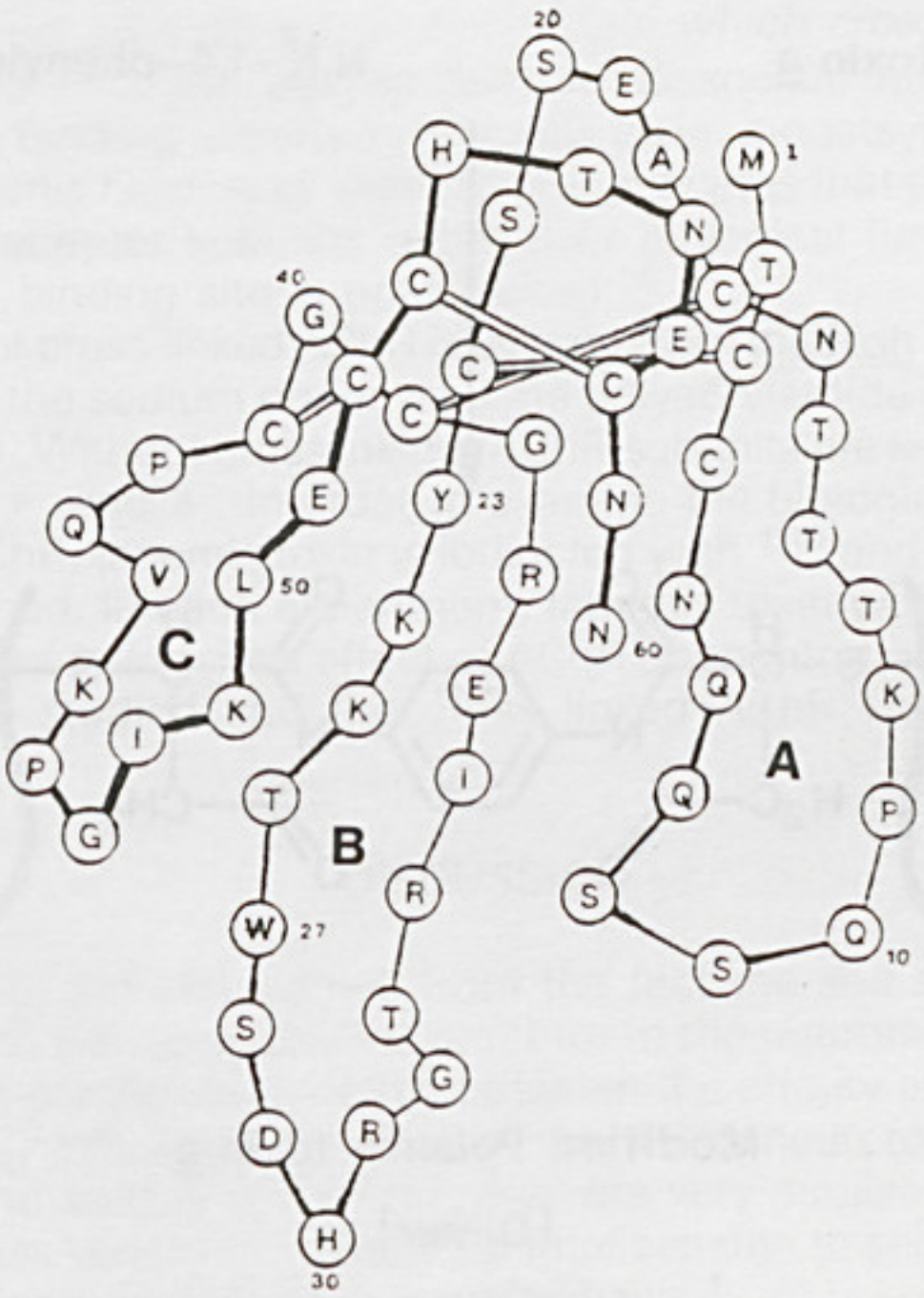


Fig. 4. Tertiary view of *Lapemis hardwickii* Lapemis toxin

In order to elucidate structure and function relationships, some amino acid residues were chemically modified and the effects of this modification on toxicity or acetylcholine receptor binding ability of the postsynaptic neurotoxins were investigated. Chemical modification of sea snake neurotoxins is summarized in Table 4.[14,19-23]

The amino acid residues in neurotoxins which are important for neurotoxic action are still not entirely clarified. Some neurotoxins contain one free SH group, while others do not. From this fact, it would be logical to assume the sulfhydryl group is not essential. This was actually proven to be the case.[20]

When N,N'-1,4-phenylenedimaleimide was used for modifying the sulfhydryl group in *pelamis* toxin, 2 mole of toxins combined with 1 mole of the reagent. With the sulfhydryl group modified, the S-H stretching vibrational band at 2578 cm^{-1} disappeared. The modification of the single sulfhydryl group did not alter the binding ability to the AChR or Toxicity (Fig. 5).[20]

Fig. 5. Chemical modification study example on neurotoxin

Disulfide bonds, however, are important in maintaining the particular toxin structure and have been shown to be essential for toxicity. When all four *disulfide bonds are reduced and alkylated, the neurotoxin loses its toxicity.*[24]

The one residue most extensively studied is tryptophan. Since it was *easily modified, it indicated that the tryptophan residue is exposed. Raman* spectroscopic analysis of a sea snake neurotoxin indicated that a single tryptophan residue is indeed exposed. The tryptophan residue lies in the im*portant loop consisting of segment 4. Modification of the tryptophan* residue induces the loss of AChR binding ability as well as the loss of toxicity.[14,19,20,22]

There is only one tyrosine residue in some sea snake neurotoxins. This residue is usually quite difficult to modify, but once it is modified, the toxicity is lost.[23]

Arginine and lysine are believed to be important, but results are not clear because sea snake neurotoxins contain several residues of these amino acids.[14]

The acetylcholine receptor connects the nerve impulse from the axon to the muscle by receiving a nerve transmitter, acetylcholine. The receptor consists of five subunits of which two are identical; it is expressed as $\alpha_2\beta\gamma\delta$. It is known that the subunit is the site for the acetylcholine binding and also for its antagonist, snake postsynaptic neurotoxin. It is generally recognized that the subunits β, γ, and δ are also essential to maintain the integrity of the acetylcholine receptor. In order to further understand the role of the subunits in the acetylcholine receptor function, the subunits *were cross-linked with dimethylsuberimidate which cross-links NH_2. The* cross-linked acetylcholine receptor does not dissociate into its components and retains the binding activity to Lapemis toxin, a postsynaptic toxin from sea snake *Lapemis hardwickii* venom. This indicates that convalently linked acetylcholine receptor subunits retain their biological function as long as the neurotoxin binding site is not blocked.[25]

Formation of cross-linked AChR is evident from the high molecular weight band shown in the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Without crosslinking, AChR subunits are separated into four subunits, α, β, γ, and δ. In order to examine the biological activity of the cross-linked AChR, lapemis toxin is iodinated with ^{125}I and the mutual binding was examined. In each experiment, lapemis toxin without crosslinking was used as the control. The effect of AChR concentration on lapemis toxin binding was studied and the cross linked AChR showed very weak binding.[26]

DISCUSSION

The origin of lethality comes from the fact the sea snake neurotoxin strongly binds to the acetylcholine receptor at the neuromuscular junction. Thus, there is a parallel relationship between the affinity and toxicity as expressed by LD_{50}. When the structure of sea snake neurotoxins is compared with that of land snakes (Elapidae), they are very similar. Fortunately, sea snake injects less venom in a bite; thus fatalities due to sea snake are fewer than those of land snakes on a per bite basis.[1]

The binding mechanism of neurotoxins to acetylcholine receptors is still

unclear. Neurotoxins are fairly small polypeptides (6800-8000 Daltons), and many of their primary structures are already known. However, owing to the large size of the multi-subunit AChR, detailed binding sites of the receptor are still less well understood.[1]

Sea snake neurotoxins show considerable homogeneity in their amino acid sequences. There are many invariant residues. That there is greater similarity in sequences within a subfamily and more differences between the two subfamilies of sea snakes is very interesting since these chemical data agree well with morphological differences between the two subfamilies.[1]

Similarity in the structures of sea snake neurotoxins are also reflected in immunologic similarities. Usually the antibody (antiserum or antivenin) produced against sea snake venom neutralizes the toxicities of other sea snake venoms quite well. Sea snake neurotoxins are not only similar among themselves but also closely resemble neurotoxins from some land snake venoms, such as those of the Elapidae (cobras and kraits). Therefore, it is advantageous to discuss the neurotoxins of Elapidae and Hydrophiidae together for a better understanding of their structure and function. The higher toxicity of Hydrophiidae venoms compared with that of Elapidae venoms is due to the higher concentration of neurotoxins in Hydrophiidae venoms.[1]

Chemical modification of each residue in the sea snake neurotoxin indicated the tryptophan-modified neurotoxin abolished the lethality and neuromuscular blocking activity, thereby indicating the essential role of tryptophan residue. It is of interest to note that both tryptophan and tyrosine residues are conserved and located at exactly the same position in the amino acid sequence regardless of the species of sea snake. When the tryptophan residue was modified with N-bromosuccinamide, the toxicity of the sea snake neurotoxins disappeared; however, they still retained the ability to bind to neurotoxin antibody. This suggested that neurotoxins can be converted into toxoids.[19]

Sea snake venoms contain potent neurotoxins that bind almost irreversibly to the postsynaptic acetylcholine receptors. Neurotoxins are the most extensively studied proteins of all the components present in sea snake venoms. Sea snake toxins are basic proteins with isoelectric points of 8.7-10. They range in molecular weight from 6,000 to 8,000. The neurotoxin is the main protein fraction although other proteins with molecular weights up to 29,500 can be found in *Pelamis platurus*. The AChR is composed of five subunitis, $\alpha_2 \beta \gamma \delta$. A neurotoxin attaches to the α subunit. Since there are 2 moles of the α subunits, 2 moles of neurotoxins attach to 1 mole of AChR. A neurotransmitter, acetylcholine (ACh), also attaches to the α subunit. When the ACh attaches to the AChR, the AChR changes conformation, opening up the transmembrane pore so that cations (Na+,K+) can pass through. By this mechanism the depolarization wave from a nerve is now conveyed to the muscle. The difference between neurotoxin and ACh is that the former's attachment does not open the transmembrane pore. As a consequence, the nerve impulse from a nerve cannot be transmitted through the postsynaptic site (Fig. 6).

Since neurotoxins are basic proteins and the AChR is acidic, it would be logical to assume that the binding of these two proteins is due to acidic and basic protein interactions. Yet, simple acidic and basic protein binding

cannot explain the extremely high affinity of these two proteins based solely on the ionic state. Therefore, there must be some other factor involved in the binding. One such factor is likely to be complementary topography of the two molecules, which allows the two proteins to lock firmly into each other.

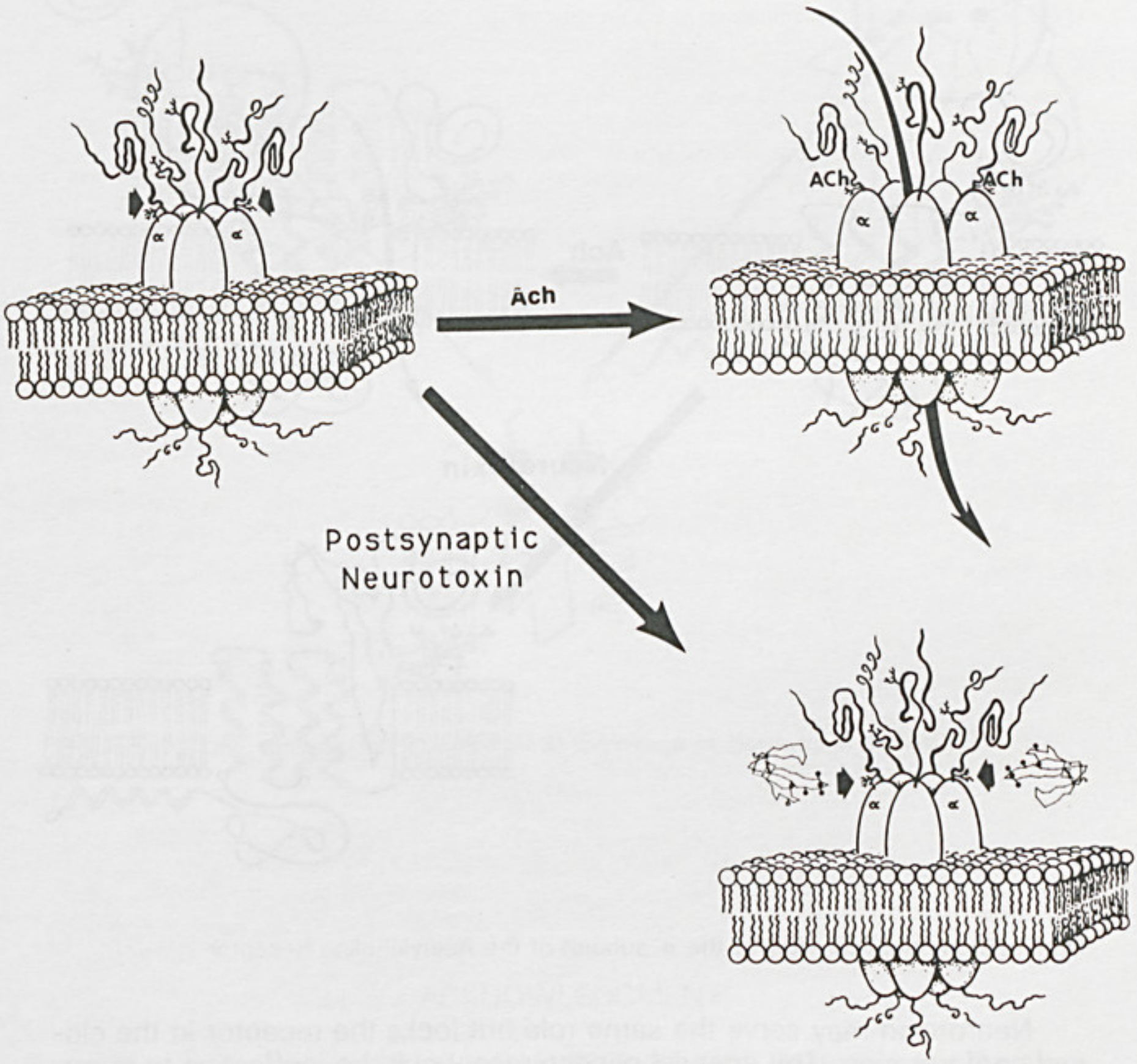


Fig. 6 Diagrammatical structure of Acetylcholine Receptor ACh (acetycholine)

The binding is very tight as stated and suggests the following working hypotheses by these authors. As mentioned the ionic state and topography of the two molecules (neurotoxin, α -AChR) play a major role in the recognition and binding. The importance of the invariant tryptophan residue of the neurotoxin may play a role in receptor recognition. The neurotoxin molecule would then lock the receptor in the closed conformation effectively blocking the receptor function to transmit the electrochemical neuronal impulse to the musculature leading to paralysis. Figure 7 diagrammatically depicts the interaction of neurotoxin with the α subunit of the AChR which is imbedded in the lipid bilayer of the muscle cell. The branched structure represents the carbohydrate moiety on the exterior surface of the muscle cell. These authors believe that there may occur a disulfide bond exchange when acetylcholine binds and the opening of the ionic channel occurs due to the conformational change in the two α subunits of the AChR.

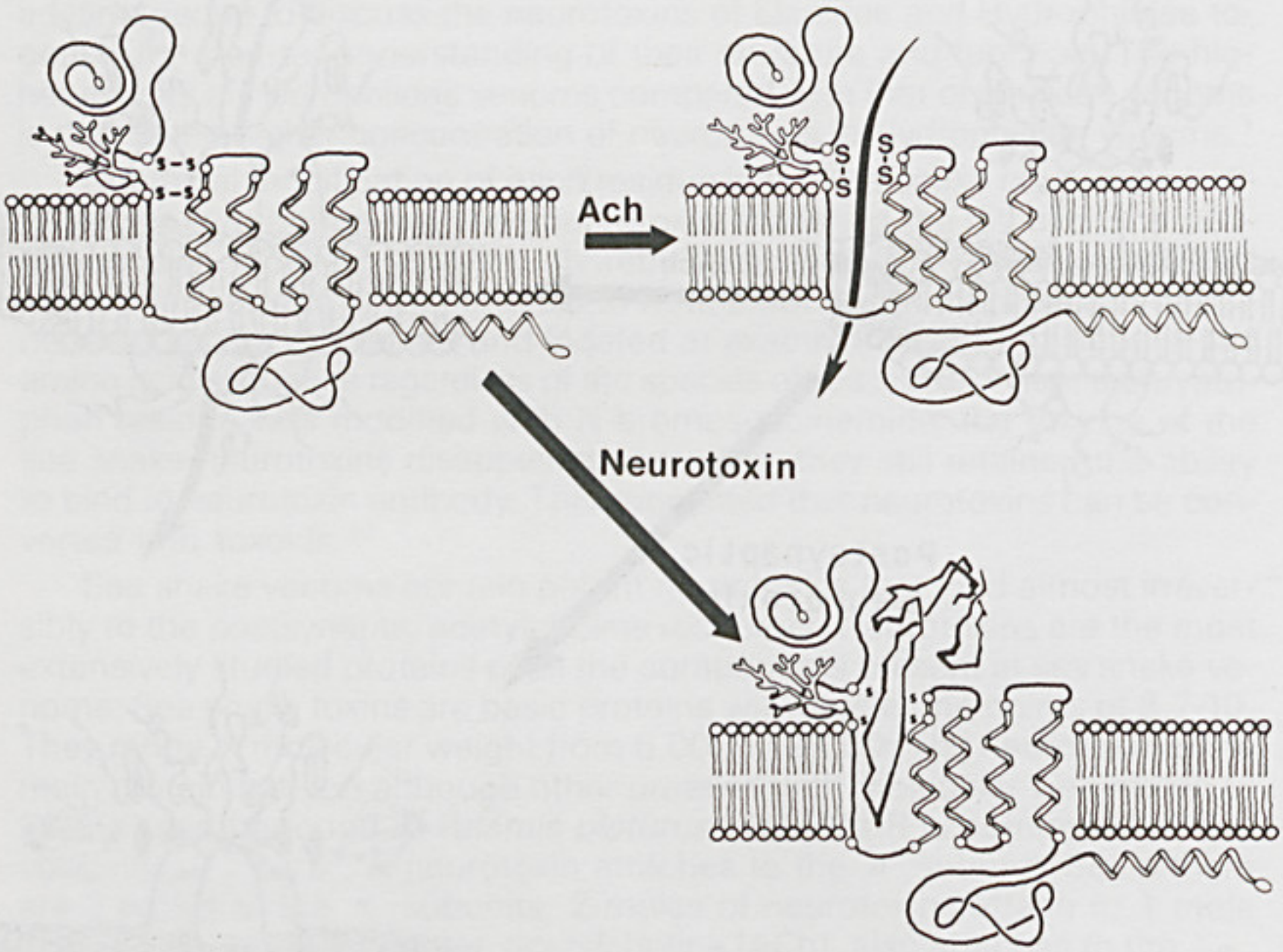


Fig. 7 Diagrammatical view of the α subunit of the Acetylcholine Receptor

Neurotoxin may serve the same role but locks the receptor in the closed conformation. The acetylcholinesterase would be ineffective to remove the neurotoxin as it does acetylcholine. This would therefore be an irreversible blockage of the receptors. The specificity and irreversibility of this action by neurotoxin probably explains the high toxicity of these molecules.

An alternative hypothesis, which is very similar to the above but may account better for the very tight binding known to occur between the AChR and neurotoxin, is that the disulfide bonds under local reducing environment actually exchange between receptor and neurotoxin molecule giving a covalent bonded receptor toxin interaction (Fig. 8). The invariant tryptophan may play a role in providing the localized reducing environment of the AChR critical disulfide bonds and the neurotoxin. Experiments are currently being designed to probe these hypothesis

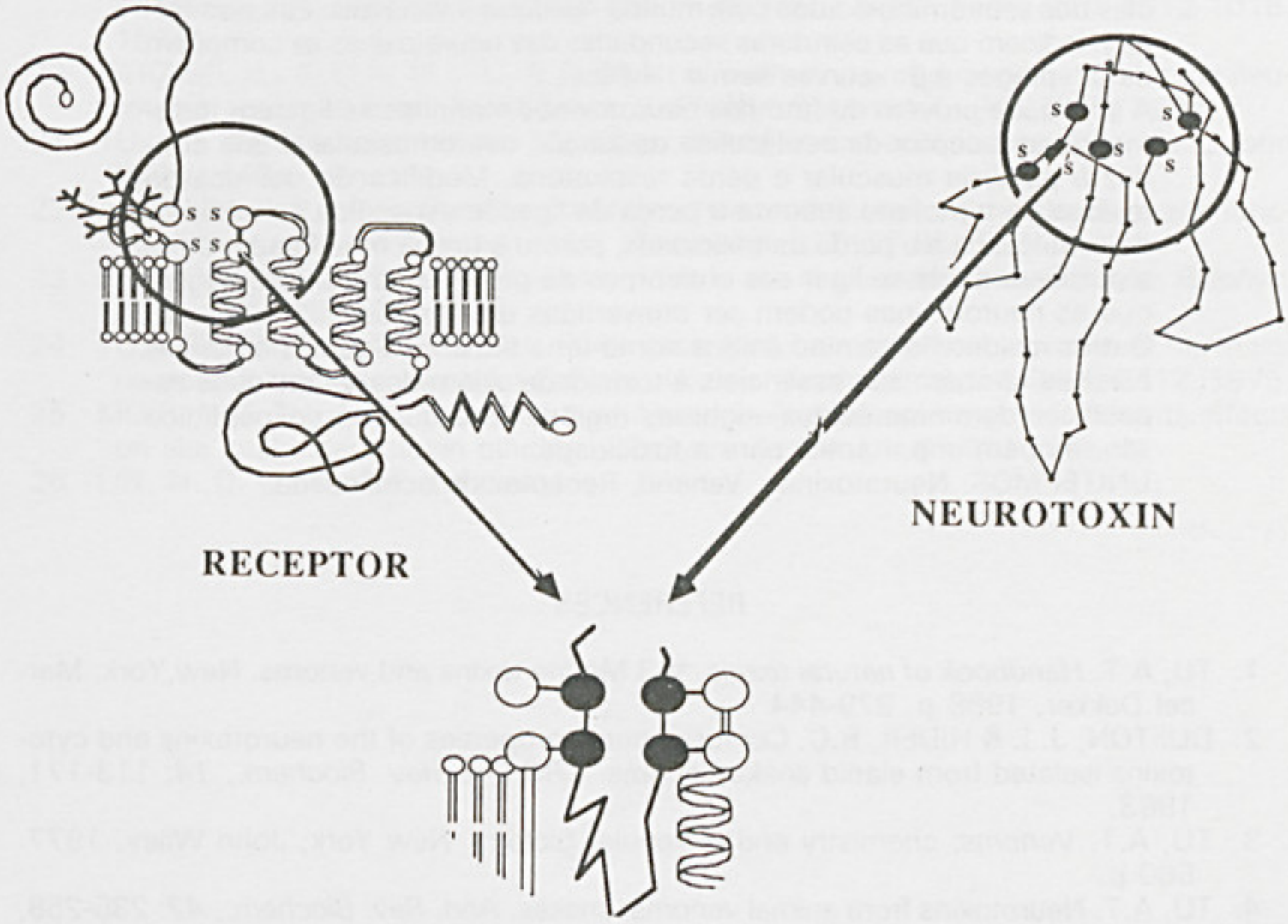


Fig. 8 Expanded view of the Disulfide (S-S) Exchange of Neurotoxin and AChR

ACKNOWLEDGMENT

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TU, A.T.; MILLER, R.A.; Biochemical studies of the sea snake neurotoxins. **Mem. Inst. Butantan, 51**(4)177 - 193, 1989.

RESUMO: Serpentes marinhas pertencentes à família Hidrofidae são serpentes adaptadas à vida aquática e são encontradas dispersas nas águas costeiras das zonas tropical e subtropical dos oceanos Indico e Pacífico.
O veneno da serpente marinha é uma mistura de várias proteínas e contém neurotoxinas potentes. A DL_{50} para o veneno da serpente marinha pode chegar até o baixo valor de 0,01 mg/kg.
As neurotoxinas pós-sinápticas do tipo I purificadas consistem de 60-62 resíduos de amino-ácidos com quatro pontes de disulfeto. São proteínas básicas com pontos isoelétricos de 9 a 10 e de pesos moleculares variando de 6.000 a 8.000 daltons. Pesquisas deste laboratório foram focalizadas sobre neurotoxinas pós-sinápticas dos venenos de *Laticauda semifasciata, Lapemis hardwickii, Pelamis platurus e Acalyptophis peronii.*
As neurotoxinas marinhas mostram grande homogeneidade das seqüências dos seus amino-ácidos com muitos resíduos invariáveis. Estudos Raman indicam que as estruturas secundárias das neurotoxinas se compõem de β -pregas e β -curvas sem α -hélices.
A letalidade provém do fato das neurotoxinas marinhas se ligarem fortemente ao receptor de acetilcolina da junção neuromuscular o que conduz à paralisia muscular e perda respiratória. Modificação química do resíduo de triptofano acarreta a perda da ligação específica ao receptor da acetilcolina e a perda da toxicidade, porém a toxina modificada retém a propriedade de se ligar aos anticorpos da proteína nativa. Isso sugere que as neurotoxinas podem ser convertidas em toxóides.
Outros resíduos de amino ácidos como uma tirosina, algumas argininas e lisinas também são essenciais à toxicidade. Além desses resíduos específicos de amino-ácidos, algumas regiões do esqueleto polipeptídico são também importantes para a toxicidade.
UNITERMOS: Neurotoxinas, Veneno, Receptor de acetilcolina.

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Mem. Inst. Butantan
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CHROMATOGRAPHIC PURIFICATION OF ANTIVENOMS AND ANTITOXINS +

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ABSTRACT: Commercial preparations of antivenoms and antitoxins produced in horses, that are essentially pure F (ab)2 immunoglobulin preparations, were submitted to ion-exchange chromatography. For anti-crotalic, anti-bothropic and anti-tetanic sera it is possible to remove 40-60% of the inactive globulins.

KEY WORDS: Antivenoms; tetanus; antisera; antitoxins.

INTRODUCTION

Antivenoms and antitoxins used for the treatment of snakebite, arthropods poisoning, diphteria or tetanus, are usually produced by immunization of horses, followed by treatment of the hyperimmune plasma with crude pepsin, and purification by heat denaturation and ammonium sulfate precipitation[3]. Previous investigations from this laboratory have shown that the product of crude pepsin digestion at pH 3.2 is a F $(ab)_2$ - like product from immunoglobulins hydrolyzed by para-pepsins present in the crude pepsin preparation, which is stable under the conditions used for heat denaturation[8].

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In the present paper we report further purification of the antisera by chromatography, that removes part of the inactive immunoglobulins.

MATERIALS AND METHODS

Hyperimmune plasma and serum: Hyperimmune plasma was prepared by the immunization of horses with snake venoms, purified diphteria or tetanus toxins. They constitute the sera supplied by the Institute for human use processed by the method of Harms.[3]

Chromatographic resins: Cibracon blue agarose and Blue Sepharose were obtained from Amicon, Pharmacia and Sigma Chemical Co, and S-Sepharose from Pharmacia.

Electrophoresis: SDS-acrylamide gel electrophoresis was carried out in 7% acrylamide[2]. Electrophoresis on cellulose acetate was carried out by the standard method used for human serum electrophoresis[4].

Immunodifusion was carried out according to Ouchterlony[9]. Identification of the heavy and light γ -immunoglobulin chains using immunodifusion was assayed by Prof. S. Kochwa at Mount Sinai School of Medicine.

Neutralizing activity: Antivenom activity was assayed by testing the effects of mixtures of a standard volume of the antiserum or one of its fractions with different (or several) dilutions of the venom in pigeons[1]. Tetanus and diphteria antitoxin activity was assayed in guinea-pigs by the methods established by the US National Institutes of Health[6,7].

Protein determination: was estimated by the absorbance at 280 nm[5].

RESULTS AND DISCUSSION

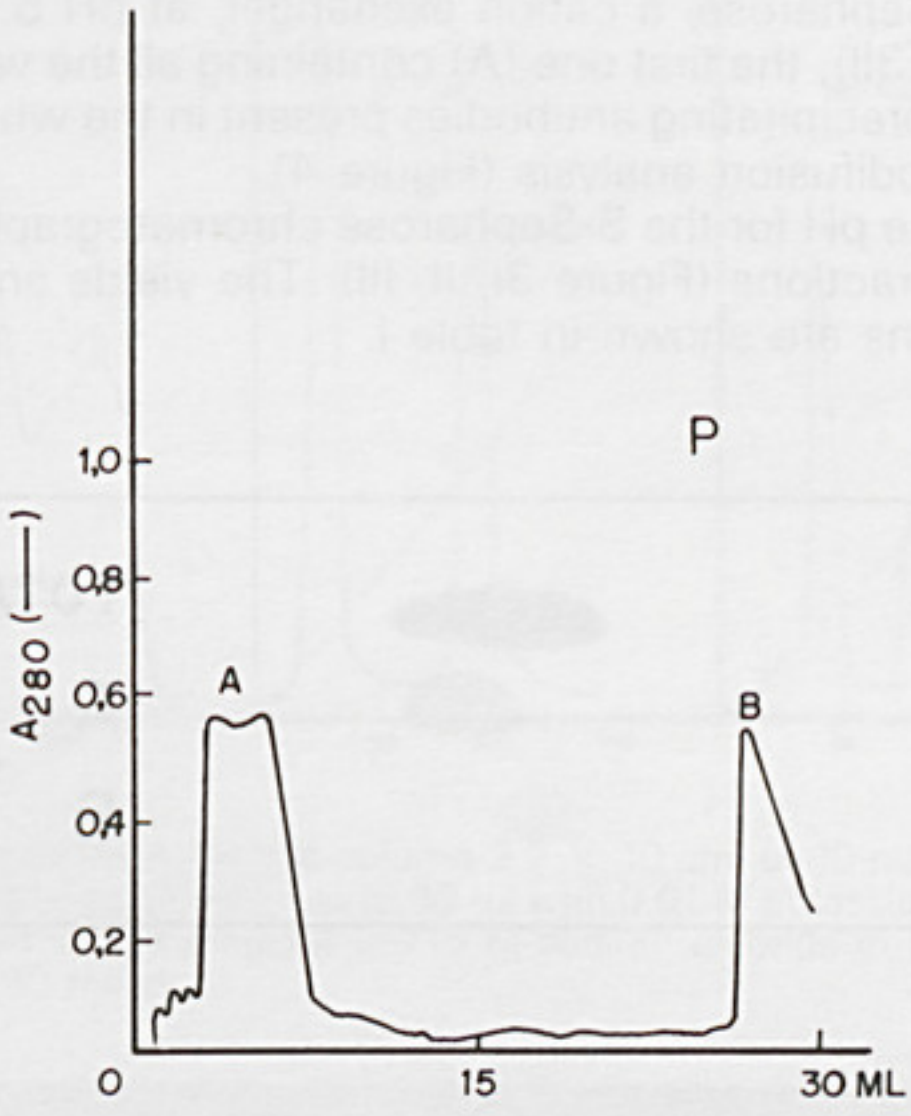
Chromatographic purification of hyperimmune anti-Crotalus venom plasma and serum

In attempting to purify anti-*Crotalus* venom plasma and serum, affinity chromatography was performed by using Cibracon blue-agarose, which is known to bind albumin but not globulins[10]. When using ''Matrex-blue'', the blue agarose produced by Amicon, two fractions were unexpectedly separated from the globulin fraction of plasma (P) and of serum (S) (Figure 1P and 1S) and only the second globulin fraction (B) had a venom neutralizing activity. When Matrex-blue was replaced by blue-agarose or blue-Sepharose from other sources, the separation of the globulins did not occur. Apparently, the difference among those resins is due to the Cibracon-blue used for their preparation, as this dye is not a chemically defined product.

On SDS-acrylamide gel electrophoresis the two serum globulin fractions seem to be identical, presenting two broad bands with an average molecular weight of 28.000-30.000 and 32.000-35.000 respectively.

Immunodifusion shows that the bands present in both fractions are heavy and light γ -globulin chains.

Electrophoresis on cellulose acetate showed that the two fractions have different mobility (Figure 2) thus differing in their electrical charges. This suggested that it would be possible to separate them by ion-exchange chro-



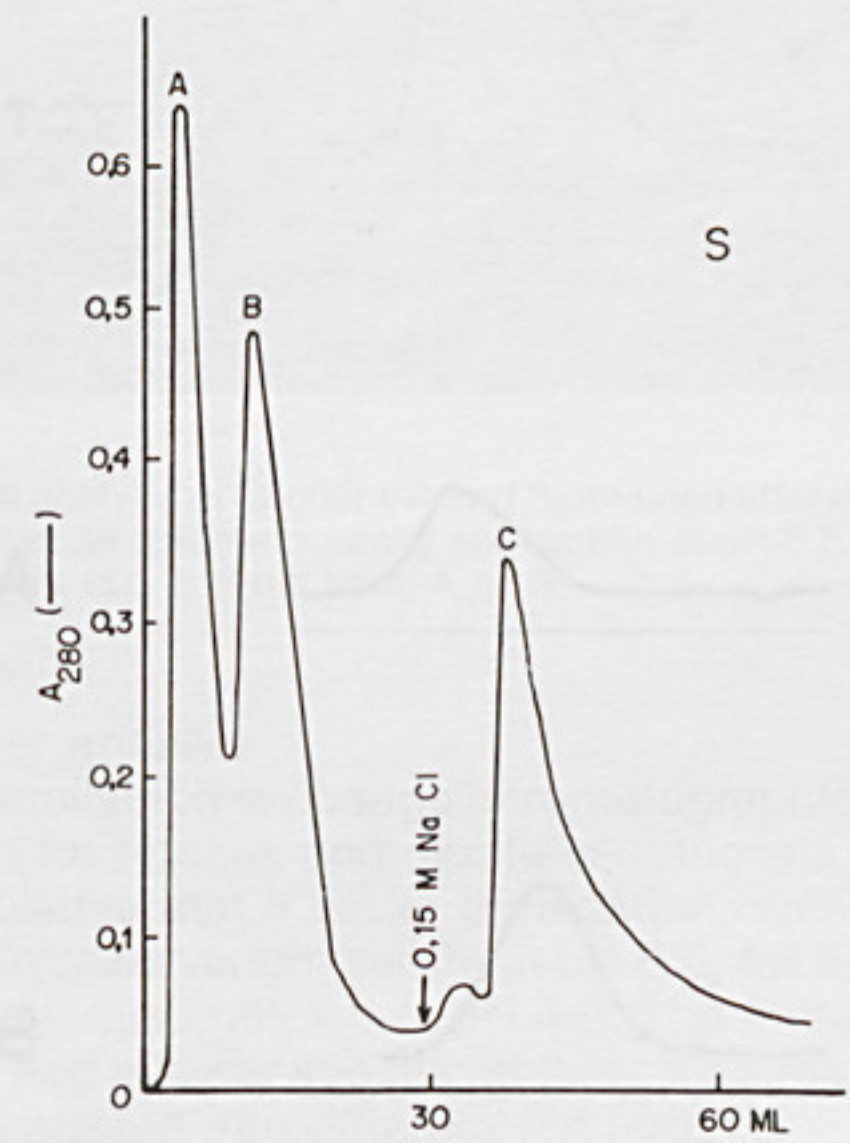


Fig. 1. Chromatography of plasma (P) or serum (S) on a Matrex-blue agarose column (2.5 x 10cm). 10 ml of horse hyperimmune plasma or serum containing 0.7 g and 0.32 g of protein respectively, were diluted to 30 ml with 1mM potassium phosphate buffer pH 6.8. Plasma and serum globulins were eluted with the same phosphate buffer, and plasma albumin with 0.15 M sodium chloride. A: globulin sub-fraction 1; B: globulin sub-fraction 2; C: albumin.

matography. S-Sepharose, a cation exchanger, at pH 5.5, separated two fractions (Figure 3II), the first one (A) containing all the venom neutralizing activity and the precipitating antibodies present in the whole serum, detectable by immunodifusion analysis (Figure 4).

By varying the pH for the S-Sepharose chromatography it was possible to obtain more fractions (Figure 3I, II, III). The yields and purifications of these preparations are shown in table I.

Fig. 2. Cellulose acetate electrophoresis of the two serum globulin fractions obtained after fractionation of anti-*Crotalus* venom serum by Matrex-blue agarose.

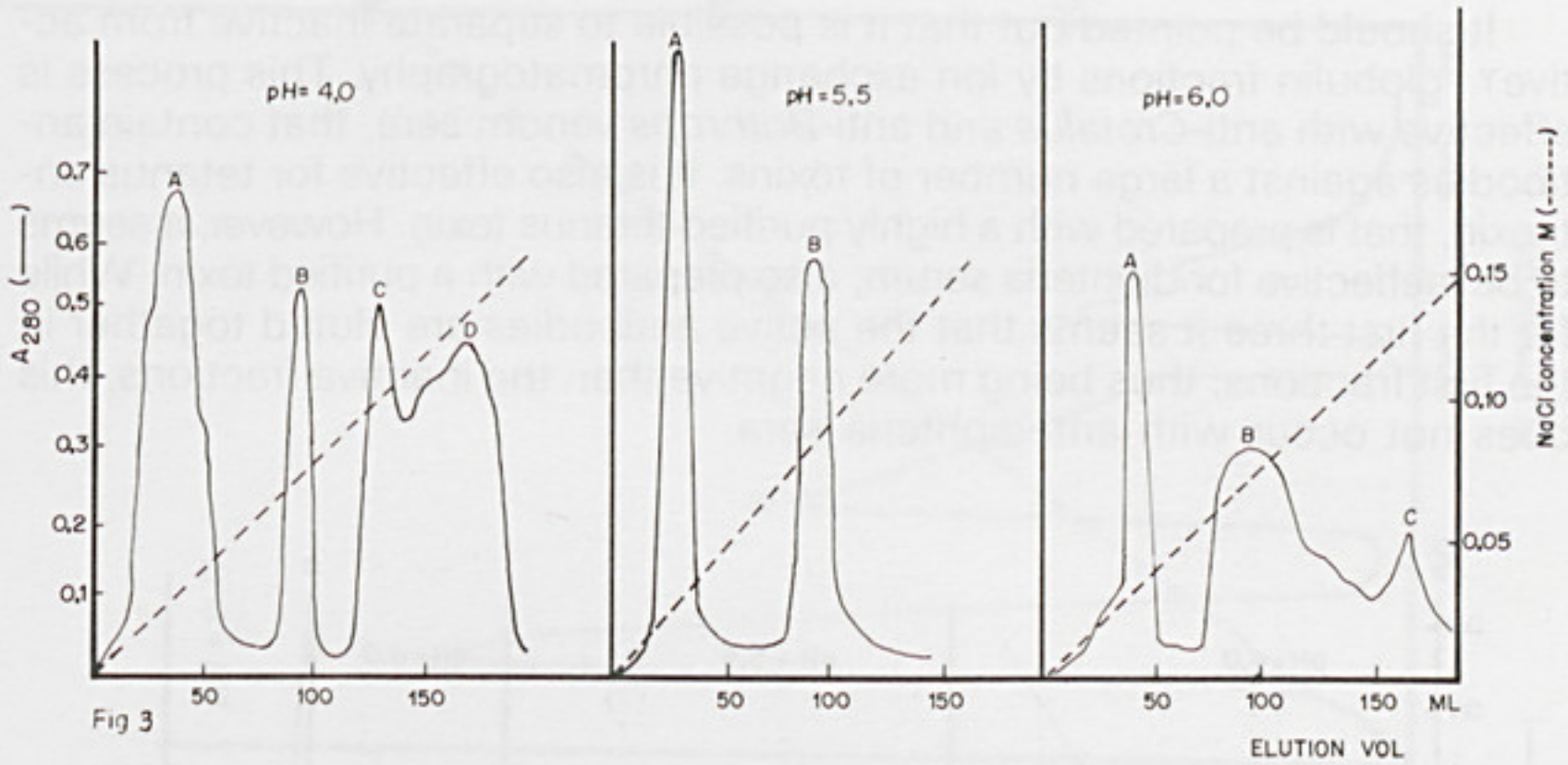


Fig. 3. Chromatogram on S-Sepharose column 2.2 × 10 cm, of 10 ml of anti-*Crotalus* serum, containing 0.32 g of protein diluted to 30 ml with 0.01 M potassium phosphate buffer at indicated pH, eluted with a gradient 0-0.15 M sodium chloride in the same buffer, for 4 hours, at a rate of 80 ml/hour.

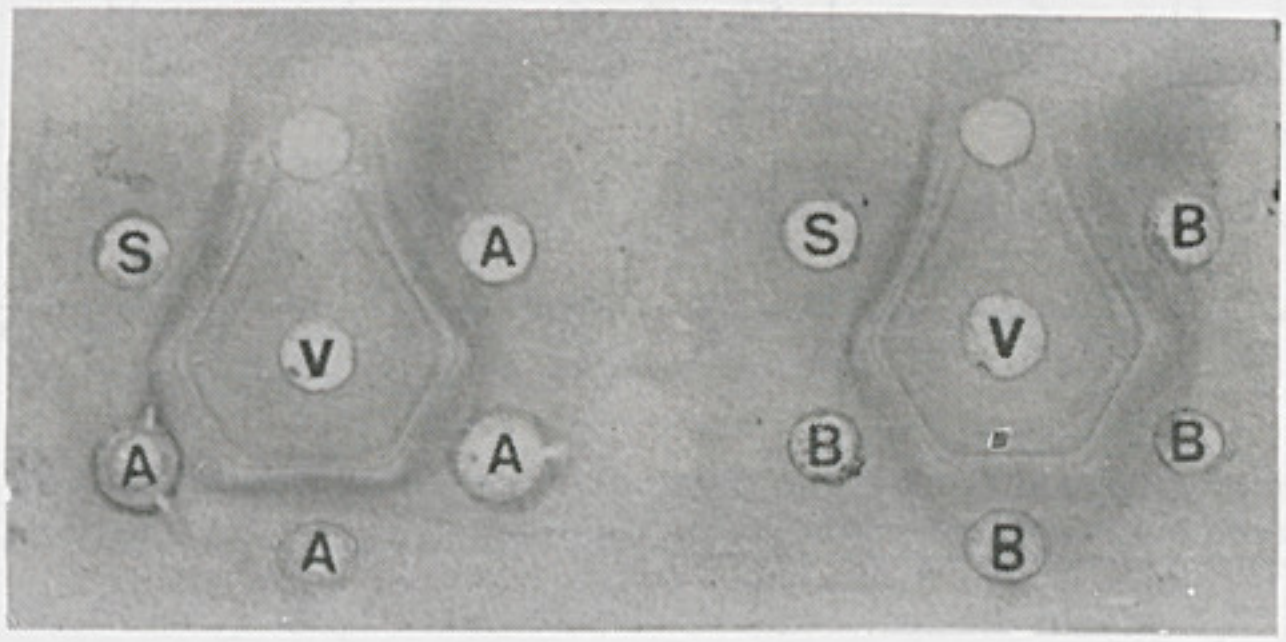


Fig. 4. Immunodifusion analysis of fractions A and B obtained after chromatography of anti-crotalic sera by S-sepharose chromatography and elution at pH 5.5 The wells contain venom (V), anti-crotalic sera (S), and fractions A or B.

Purification of other antisera

Table I shows similar ion exchange chromatographies for anti-*Bothrops* venom serum, and for tetanus and diphteria antitoxins (figures 5, 6, 7). It is interesting to observe that a better purification, with a good yield, was obtained for anti-*Crotalus* venom serum at pH 6.0, for anti-*Bothrops* serum at pH 5.5 and for tetanus antitoxin at pH 4.0. The fractions obtained from diphteria antitoxin had a lower specific activity than the whole serum, and the activity was scattered into different fractions.

The treatment of snakebites requires the administration of 50 to 100ml of antivenom. As shown in this paper, almost half of the protein administered is inactive and it can be removed by a single column chromatography, allowing not just to remove inactive foreign proteins, but to reduce the volume of the antivenom administration.

It should be pointed out that it is possible to separate inactive from active γ -globulin fractions by ion exchange chromatography. This process is effective with anti-*Crotalus* and anti-*Bothrops* venom sera, that contain antibodies against a large number of toxins. It is also effective for tetanus antitoxin, that is prepared with a highly purified tetanus toxin. However, it seems to be ineffective for diphteria serum, also prepared with a purified toxin. While for the first three it seems that the active antibodies are eluted together in the first fractions, thus being more negative than the inactive fractions, this does not occur with anti-diphteria sera.

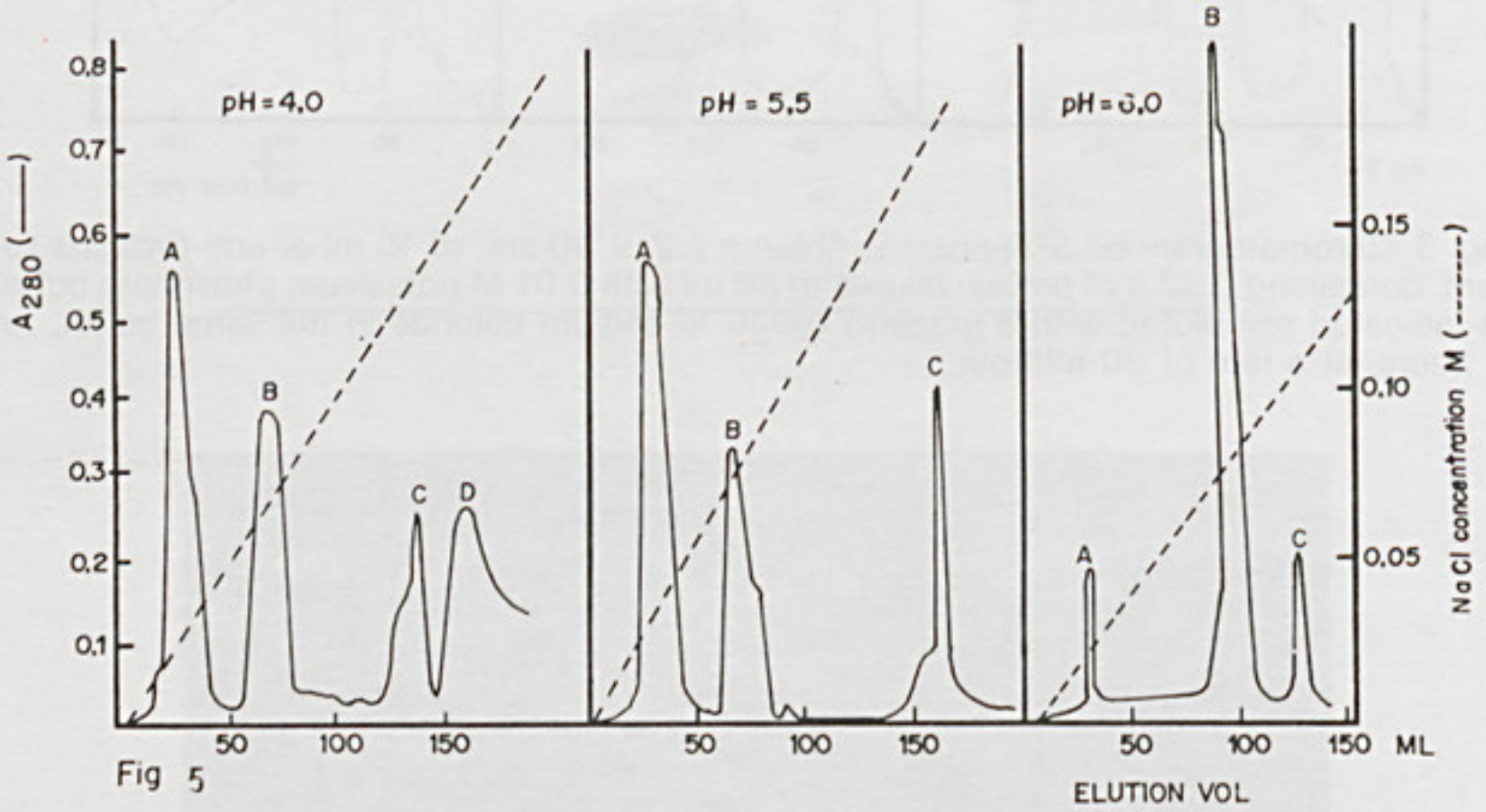


Fig. 5. Chromatography of 10 ml of anti-*Bothrops* venom serum containing 0.2 g of protein under the same conditions as described in Figure 3.

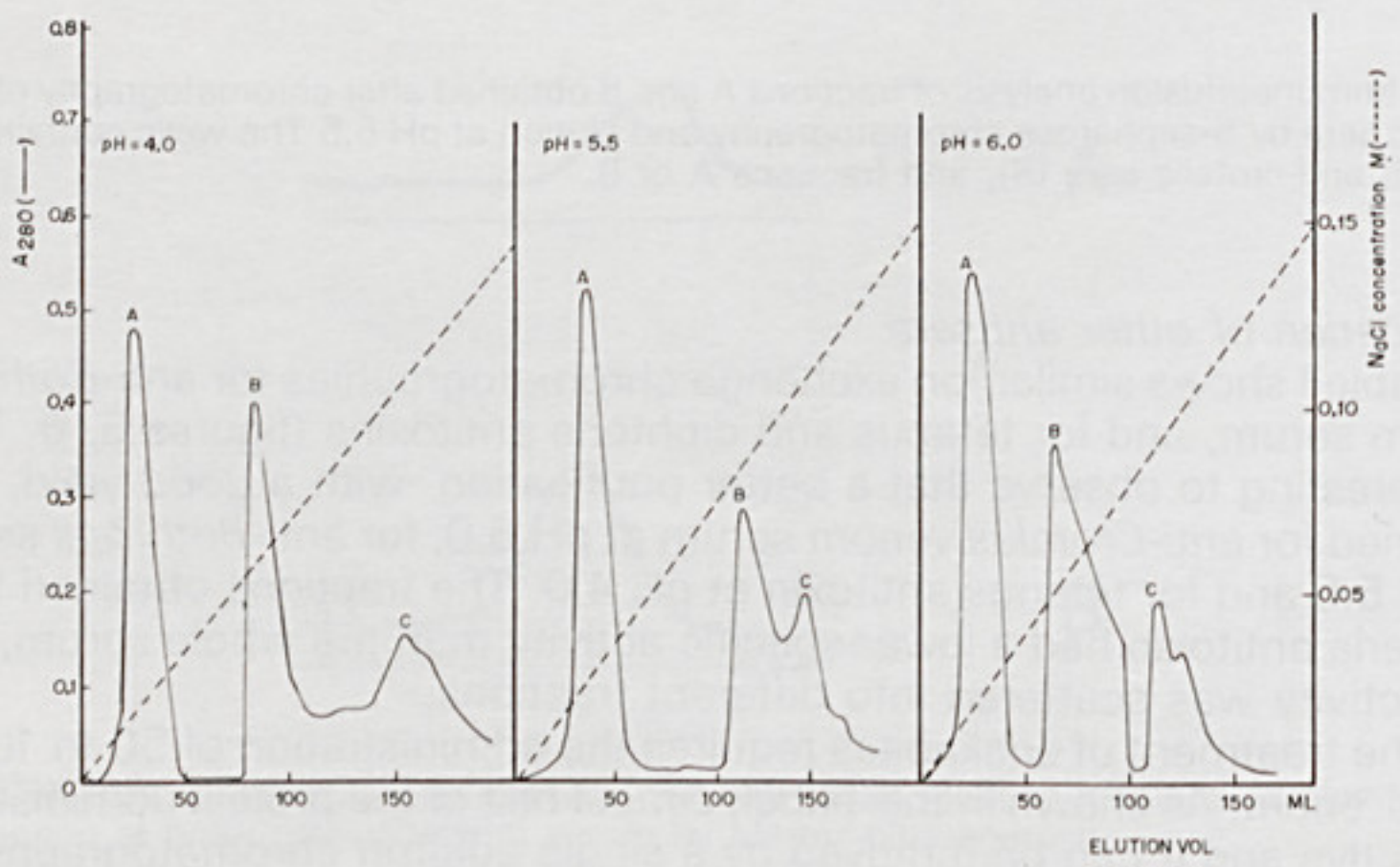


Fig. 6. Chromatography of 10 ml of anti-tetanus toxin serum containing 0.60 g of protein under the same conditions as described in Figure 3.

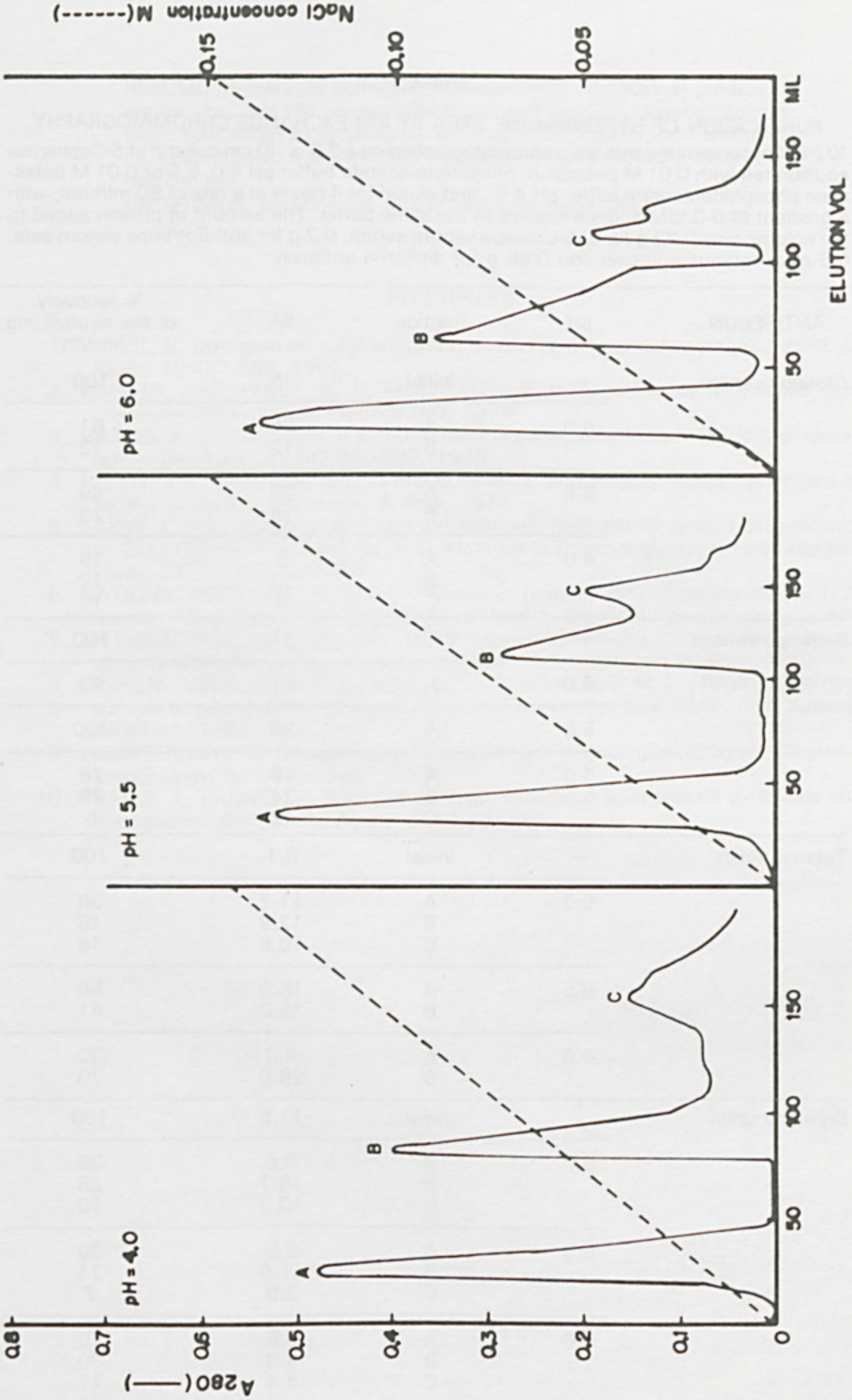


Fig. 7. Chromatography of 10 ml of anti-diphteria toxin serum containing 0.68 g of protein under the same conditions as described in Figure 3.

TABLE 1

PURIFICATION OF HYPERIMMUNE SERA BY ION-EXCHANGE CHROMATOGRAPHY

10 ml of hyperimmune sera were chromatographed on a 2.5 × 10 cm column of S-Sepharose equilibrated with 0.01 M potassium phosphate-acetate buffer pH 6.0, 5.5 or 0.01 M potassium phosphate-acetate buffer pH 4.0.. and eluted for 4 hours at a rate of 80 ml/hour, with a gradient of 0-0.15M sodium chloride in the same buffer. The amount of protein added to the column was 0.32 g for anti-*Crotalus* venom serum, 0.2 g for anti-*Bothrops* venom sera, 0.6 g for tetanus antitoxin and 0.68 g for diphteria antitoxin.

ANTISERUN	pH	fraction	SA	% recovery of the neutralizing activity
Crotalus venom	—	initial	15	100
	6.0	A	25	81
		B	12	12
	5.5	A	28	49
		B	14	23
	4.0	A	6	19
		B	12	12
		C	3	10
Bothrops venom	—	initial	11	100
	6.0	A	11	93
	5.5	A	30	100
	4.0	A	19	28
		B	24	25
		C	12	5
Tetanus toxin	—	initial	8.1	100
	6.0	A	11.7	38
		B	12.9	18
		C	10.8	18
	5.5	A	15.0	50
		B	15.0	41
	4.0	A	9.0	30
		B	28.0	70
Diphteria toxin	—	initial	17.5	100
	6.0	A	9.6	30
		B	16.7	25
		C	10.3	10
	5.5	A	9.5	30
		B	11.9	21
		C	3.8	7
	4.0	A	6.8	15
		B	8.0	30
		C	4.4	17

* Fractions not tabulated had less that 30%
SA — specific activity is the venom neutralizing activity/A_{280nm}

DIAS, W; de O. et al. Chromatografic purification of antivenoms and antitoxins. **Mem. Inst. Butantan, 51**(4):195-203, 1989.

RESUMO: Preparação comercial de antivenenos e antitoxinas produzidas em cavalos, que são preparações puras de F (ab)2 de imunoglobulinas, foram submetidas à cromatografia de troca iônica. Para os soros anticrotálico, antibotrópico e antitetânico é possível remover 40-60% de globulinas inativas.

UNITERMOS: antivenenos, tétano, anti-soro, antitoxinas.

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SARAFOTOXINS, A NEW GROUP OF CARDIOVASCULAR MODULATORS FROM SNAKE VENOM

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Dan GRAUR
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ABSTRACT: A new group of toxins from the venom of the snake *Atractaspis*, the sarafotoxins, are highly homologous with the endothelins that originate from the endothelium of mammalian blood vessels. Both groups of compounds are 21 amino acid peptides that affect the cardiovascular system and also bind to various regions of the brain. The sarafotoxins may have originated from endogenous modulators of the cardiovascular system that evolved into toxins in the venom glands of *Atractaspis*.
KEY-WORDS: Sarafotoxins, Endothelins, *Atractaspis*, Snake Venom.

Several components of snake venoms served as tools for the elucidation of natural processes in various tissues. The best known components are the postsynaptic neurotoxins from Elapidae venoms — the alpha-bungarotoxins and alpha-cobrotoxins. These toxins bind strongly to the acetylcholine receptors of the neuromuscular junction of striated muscles and were used in order to identify, isolate and characterize these receptors (Albuquerque *et al.*[1]).

During the last year (1988), several isotoxins from the venom of the Burrowing Asps genus *Atractaspis*, were shown to mimic natural products from mammalian blood vessels, the endothelins (Bdolah *et al.*,[4,5] Wollberg *et al.*,[15]).

The Burrowing Asps are now considered to belong to a separate family, the Atractaspididae (Fig. 1), that differ considerably from the other veno-

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mous and non-venomous snakes in many respects (Underwood and Kochva,[14]). The venom of *Atractaspis* has a characteristic composition and contains both high and low molecular weight components. Some of these, hemorrhagin, protease and phospholipase A_2 are found also in the venoms of other snakes (Ovadia,[12]). However, about 1/3 of the venom consists of a new type of highly lethal low molecular weight toxins (Fig. 2; Kochva *et al.*,[11]). These toxins were first labeled S_5 and S_6, in order of their elution from a G-50 Sephadex column; they showed one and two bands, respectively, in acrylamide gel electrophoresis. A preliminary sequence of S_5 was later identified as sarafotoxin c, while S_6 was found to contain mainly sarafotoxins a and b. These three toxins were named after the common Hebrew name of the Israel Burrowing Asp, SARAF Ein-Gedi and were shown to be highly homologous isotoxins that contain 21 amino acid residues with two disulphide bridges (Fig. 3; Takasaki *et al.*,[13]). A search of the several protein and nucleic acid sequence data banks yielded no meaningful similarities with any of the published sequences. However, the mammalian endothelins, that are also composed of 21 amino acids, are highly homologous with the venom sarafotoxins (Yanagisawa *et al.*,[17], Graur *et al.*[6]).

Fig. 1. *Atractaspis engaddensis*, Haas 1950.

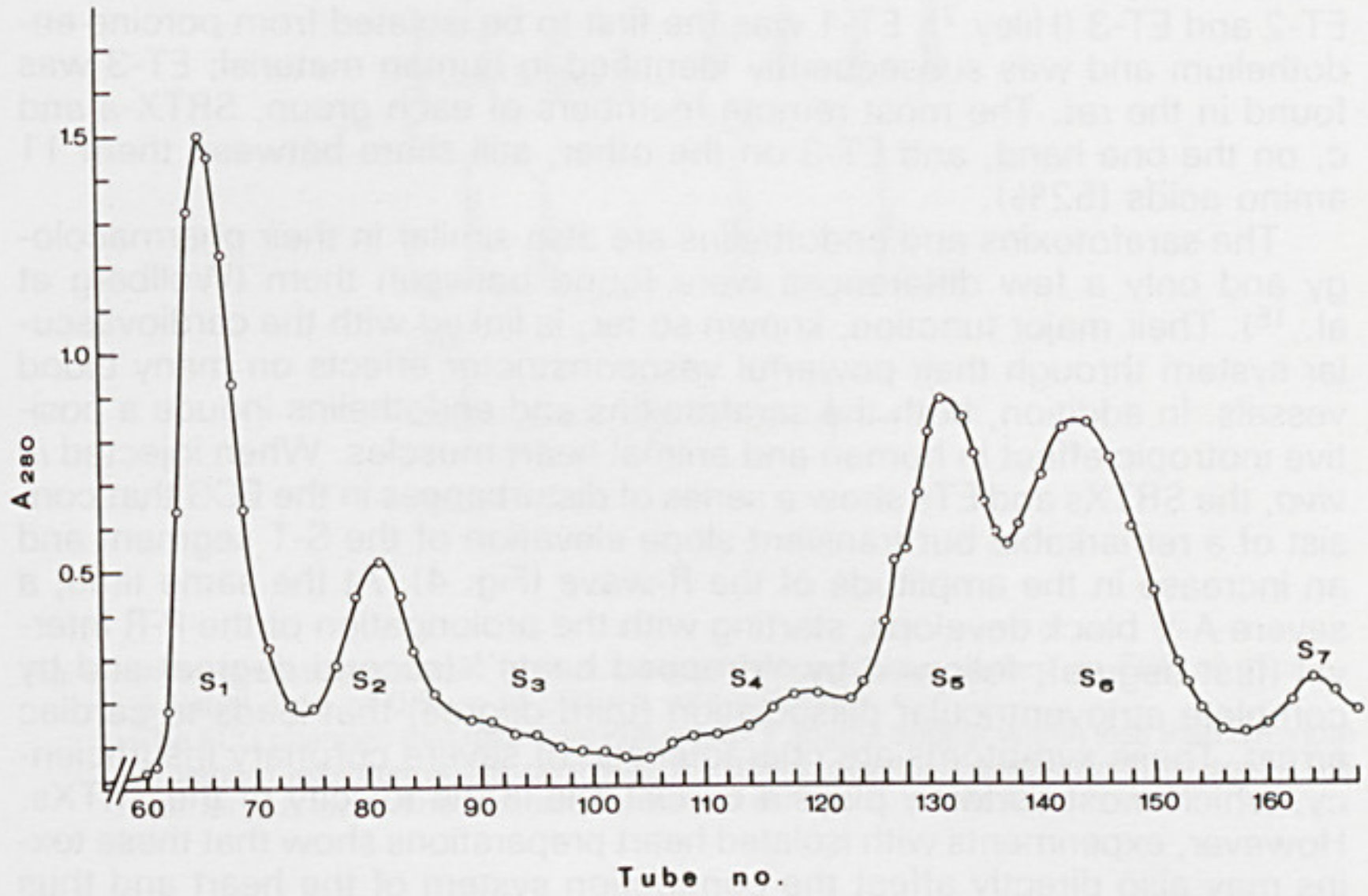

Fig. 2. Sephadex G-50 profile of *Atractaspis engaddensis* venom. Ten to 30 mg lyophilised venom were dissolved in 1 ml of 0.05 M NH_4 CO_3, applied onto a 0.9 × 152 cm column, and eluted with the same solution at a rate of 2ml/hr, in 1 ml fractions. S_1 contains L-amino acid oxidase and hemorrhagin; S_2 contains protease(s); S_3 contains phospholipase A_2; S_5 contains mainly SRTX-c; S_6 contains SRTX-b and SRTX-a.

SARAFOTOXIN-ENDOTHELIN SEQUENCES

Cys-Ser-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp	SRTX-a
Cys-Ser-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Tyr-Phe-Cys-His-Gln-Asp-Val-Ile-Trp	SRTX-b
Cys-Thr-Cys-Asn-Asp-Met-Thr-Asp-Glu-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp	SRTX-c
Cys-Thr-Cys-Phe-Thr-Tyr-Lys-Asp-Lys-Glu-Cys-Val-Tyr-Tyr-Cys-His-Leu-Asp-Ile-Ile-Trp	ET-3
Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp	ET-1
Cys-Ser-Cys-Ser-Ser-Trp-Leu-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp	ET-2

1 5 10 15 20

Fig. 3.Amino acid sequences of sarafotoxins and endothelins (Takasaki et. al.[13] 1988; Yanagisawa et al.[17], 1988).

To date, three endothelins were identified in the human gene, ET-1, ET-2 and ET-3 (Hiley.[7]). ET-1 was the first to be isolated from porcine endothelium and was subsequently identified in human material; ET-3 was found in the rat. The most remote members of each group, SRTX-a and c, on the one hand, and ET-3 on the other, still share between them 11 amino acids (52%).

The sarafotoxins and endothelins are also similar in their pharmacology and only a few differences were found between them (Wollberg et al.,[15]). Their major function, known so far, is linked with the cardiovascular system through their powerful vasoconstrictor effects on many blood vessels. In addition, both the sarafotoxins and endothelins induce a positive inotropic effect in human and animal heart muscles. When injected *in vivo*, the SRTXs and ETs show a series of disturbances in the ECG that consist of a remarkable but transient slope elevation of the S-T segment and an increase in the amplitude of the R-wave (Fig. 4). At the same time, a severe A-V block develops, starting with the prolongation of the P-R interval (first degree), followed by ''dropped beats'' (second degree) and by complete atrioventricular dissociation (third degree) that leads to cardiac arrest. These symptoms are characteristic of severe coronary insufficiency, which most certainly plays a crucial role in the toxicity of the SRTXs. However, experiments with isolated heart preparations show that these toxins may also directly affect the conduction system of the heart and thus contribute for their toxicity (Wollberg *et al.*[16]).

When injected *i.v.* into mice, both SRTX-b and ET-1 are highly toxic with an approximate LD_{50} of 0.015 μg/g (Bdolah *et al.*[4]).

In addition, both SRTXs and ETs bind to and compete for the same receptors and induce phosphoinositide hydrolysis in the heart and brain (Ambar et al.,[2]; Ambar et al.,[3]; Kloog et al.[8]; Kloog et al.[9]).

The structural and functional similarities between SRTXs and ETs have elicited extensive experimental work of a comparative nature, with the SRTXs being used as probes for the elucidation of the role of ETs in the regulation of blood pressure and other physiological and pharmacological phenomena of the cardiovascular system. Only time will tell whether the SRTXs will achieve the level of scientific importance of their elapid counterparts, the postsynaptic neurotoxins.

The high level of homology between the SRTXs and ETs suggests a common phylogenetic origin for the endothelin/sarafotoxin family of peptides (Fig. 5). Although endothelins are yet to be identified in non-mammalian vertebrates, they are most probably present in snakes. From the evolutionary point of view, it appears that a product already found in other tissues has evolved and adapted to a new function in the venom glands to help in the hunting for food. A similar phenomenon was recognised in other venom components, where enzymes, such as proteases and phospholipase A_2, evolved into toxic hemorrhagins and presynaptic neurotoxins, respectively (Kochva,[10]).

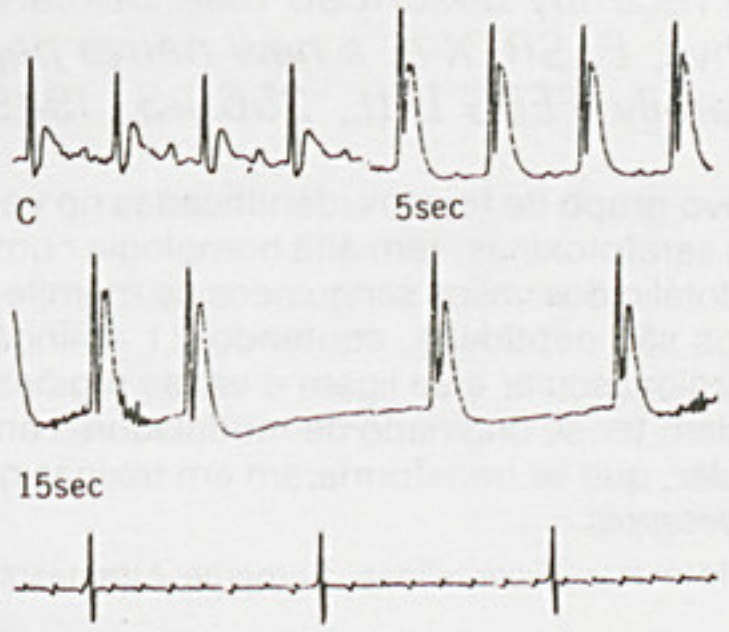

Fig. 4. Influence of sarafotoxin from *Atractaspis engaddensis* venom on ECG of mice. A lethal dose of 0.5 ug of SRTX-b was injected *i. v.* The ECG changes that resemble Prinzmetal's angina appear within less than a minute of venom injection and a complete A-V block develops gradually. Time scale: 0.5 sec for the lower line; 0.1 sec for all the others; C: control.

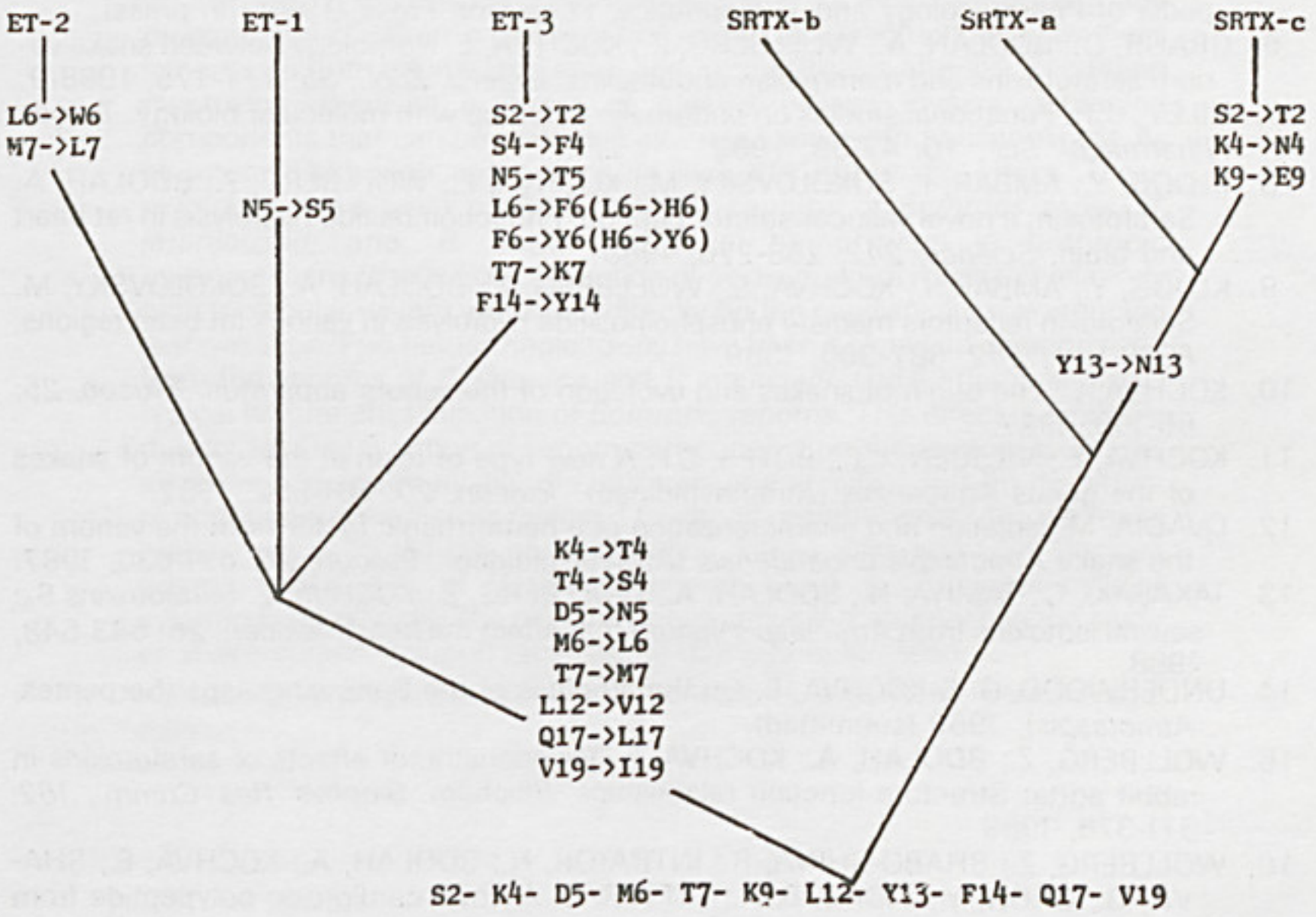

Fig. 5. Cladogram of venom sarafotoxins and mammalian endothelins. Substitutions are enumerated on the branches. Equally probable alternative substitutions are shown in parentheses.

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Note added in proof: Two additional members of the endothelin/sarafotoxin family were recently described (see Bdolah, E.; Wollberg, Z.; Fleminger, G.; Kochva, E. SRTX-d, a new native peptide of the endothelin/sarafotoxin family. FEBS Lett., 256:1-3, 1989.

RESUMO: Um novo grupo de toxinas identificadas no veneno da serpente *Atractaspis*, as sarafotoxinas, tem alta homologia com as endotelinas, originárias do endotélio dos vasos sanguíneos de mamíferos. Os dois grupos de compostos são peptídeos, contendo 21 aminoácidos, que afetam o sistema cardiovascular e se ligam a várias regiões do cérebro. As sarafotoxinas podem ter-se originado de moduladores endógenos do sistema cardiovascular, que se transformaram em toxinas nas glândulas venenígeras da *Atractaspis*.

UNITERMOS: Sarafotoxinas, Endotelinas, Serpente Atractaspis-veneno.

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LOCAL TISSUE DAMAGE INDUCED BY *BOTHROPS* SNAKE VENOMS. A REVIEW

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Bruno LOMONTE

ABSTRACT: This review focuses on the local effects induced by *Bothrops* venoms. These envenomations are characterized by myonecrosis, hemorrhage and edema which develop rapidly after venom inoculation. Myonecrosis is caused (a) directly, due to the direct action of myotoxins on the plasma membrane of muscle cells, and (b) indirectly, through the development of ischemia resultant from drastic alterations induced by these venoms on both microvasculature and intramuscular arteries. Regarding myotoxins, there is a group of closely related muscle damaging components that can be classified as ''myotoxins with phospholipase A_2 structure'', although some of them lack this enzymatic effect. These myotoxins have been purified from the venoms of *Bothrops asper, B. jararacussu,* and. *B. nummifer.* Local hemorrhage in bothropic envenomations results from the action of acidic metalloproteins of relatively high molecular weight which act directly on the capillary vessels inducing extravasation. Five hemorrhagic toxins have been isolated an characterized from the venoms of *B. jararaca* and *B. neuwiedi.* Local edema is also a typical feature after injection of *Bothrops* venoms. This effect is probably due to: (a) Direct action of venom components on the microvasculature, increasing the permeability of capillaries and venules, and (b) the effect of endogenous mediators released by venom components. *Among* these mediators are histamine, prostaglandins, kinins, C3a and C5a. Besides edema, inoculation of *Bothrops* venoms elicits a prominent cellular inflammatory infiltrate. The need of a comprehensive approach in the study of snake venom-induced local tissue damage is stressed.

KEY WORDS: *Bothrops* venoms, myonecrosis, myotoxins, hemorrhage, edema.

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INTRODUCTION

The large majority of snakebites in Latin America are inflicted by species classified in the genus *Bothrops* [5,8,65]. Despite the existence of evident intraspecific and interspecific variations in the composition and pharmacological activities of their venoms [3,15,30] they induce a qualitatively similar pathophysiological picture, characterized by: (a) Immediate and prominent local tissue damage, i.e. myonecrosis, hemorrhage and edema [17,65]; (b) cardiovascular alterations, especially hemorrhage, and hypovolemic shock[2,65]; (c) coagulation disorders, most frequently defibrination [2,5,65]; and (d) renal alterations which might evolve into acute renal failure[1,2,69].

If antivenom administration is initiated rapidly after envenomation, neutralization of systemic effects is usually achieved successfully, but neutralization of local tissue damage is a more difficult task. In a number of snakebite cases, lack of neutralization of local effects results in permanent sequelae, i.e. tissue loss. Due to the relevance of local effects in envenomations induced by snakes of the genus *Bothrops*, several research groups have studied this problem from different perspectives. In this work we will review their findings, and some conclusions regarding the pathogenesis of local tissue damage by these venoms will be presented.

LOCAL MYONECROSIS

Myonecrosis is a common consequence of envenomations by species of *Bothrops* [2,5,56,65]. Experimental studies have observed this activity in the venoms of *B. asper, B. nasuta, B. nummifer, B. godmani, B. lateralis, B. ophryomegas B. pičadoi* and *B. schlegelii* from Costa Rica [14,16,28,44,68], and of *B. jararaca, B. neuwiedi* [44], *B. jararacussu* [58] and *B. alternatus* [57] from Brazil.

The development of muscle damage has been studied with the venoms of *B. asper* [16,19], *B. jararacussu* [58] and *B. alternatus* [57].

In the first two cases myonecrosis was evident from the beginning, and necrotic fibers were characterized by the presence of amorphous clumped masses of myofibrils alternating with empty spaces. In the case of *B. alternatus* venom, necrotic fibers presented a ''waxy hyaline appearance''.

Homma and Tu [28] proposed a classification of venom-induced myonecrosis based on the morphology of the necrotic fibers 24 hr after injection of the venoms. ''Myolytic'' type of necrosis was characterized by fibers where myofibrillar material appeared clumped and alternating with empty spaces in the cytoplasm. ''Coagulative'' type of necrosis, on the other hand, was typified by cells whose myofibrillar material had a more hyaline appearance and a homogeneous distribution. According to these authors, the venoms of *B. atrox* and *B. schlegelii* induced a ''myolytic'' necrosis, whereas those of *B. nasuta* and *B. picadoi* induced a ''coagulative'' myonecrosis; the venom of *B. nummifer* caused a ''mixed'' myonecrosis, i.e. presenting both myolytic and coagulative necrotic fibers. In 1980, Gutiérrez and Chaves [14] used these criteria to characterize myonecrosis induced by several Costa Rican crotaline venoms.

As further studies clearly demonstrated, this classification is an over-

simplification of a complex and dynamic pathologic phenomenon. For instance, when muscle tissue was taken at various time intervals after i.m. injection of *B. asper* venom, it was observed that the morphology of necrotic fibers changed [19]. In the first 3 hr there was a predominance of necrotic fibers with ''clumped'' myofibrillar material alternating with cytoplasmic spaces devoid of myofilaments (''myolytic fibers''). However, after the sixth hour, the large majority of necrotic fibers contained a more hyaline cytoplasm, with myofibrillar material distributed in a more uniform fashion in the cellular space [19]. Thus, the morphological type of necrosis is different depending upon the time of tissue sampling. Similar findings have been made recently with the venoms of *Naja naja, Crotalus viridis* and *Crotalus atrox* [51].

Despite the lack of validity of this ''myolytic'' vs ''coagulative'' classification of myonecrosis, experimental work performed with the venoms of *B. jararacussu* [58], *B. asper* [19] and *B. alternatus* [57] strongly suggested that there are, at least, two different ways by which *Bothrops* venoms affect muscle cells: (a) directly, by the action of ''myotoxins'' which probably affect the integrity of skeletal muscle plasma membrane [19,57]; (b) indirectly, through an ischemic condition that develops in skeletal muscle secondarily to the disruptive action of venoms on the vasculature [19,57]. Morphologically, cells affected by myotoxins initially presented clumping of myofibrils and subsequently became hyaline in appearance[19,27,58]. In contrast, other necrotic cells, probably affected by ischemia, presented a hyaline morphology from the beginning[19,57]. Similar observations were made by Ownby and Colberg[51], with the venoms of *Crotalus atrox* and *Crotalus viridis,* describing these cells as ''moth-eaten'' on the basis of their morphology in sections made from plastic-embedded tissue.

In the past, the study of myonecrosis induced by snake venoms was limited by the lack of reliable quantitative assays to estimate the extent of muscle damage. Moreover, the fact that only histology allowed these studies precluded many laboratories to isolate and characterize myotoxins. In recent years, several techniques have been used in the quantitation of venom-induced myonecrosis, such as: (a) quantitation of serum levels of the enzyme creatine kinase and, more specifically, of the isozyme CK-MM[10,16,18,44,47,54]; (b) quantitation of the release of creatine kinase *in vitro* from preparations of skeletal muscle incubated with venoms [23,45]; (c) quantitation of the residual content of creatine kinase in muscle injected with venoms or toxins [26]; and (d) quantitative histological estimation of muscle damage, by counting the number of necrotic and surviving cells [33,55].

MUSCLE DAMAGE DUE TO THE DIRECT ACTION OF MYOTOXINS

Five myotoxins have been purified to homogeneity from *Bothrops* venoms: *B. asper* myotoxin I [18], *B. asper* myotoxin II [35], *B. nummifer* myotoxin [22], and two myotoxins from the venom of *B. jararacussu,* one of which was named ''bothropstoxin'' [29]. They had molecular weights of 13,000-16,000 and basic isoelectric points, showing similarities in their amino acid composition. When analyzed by polyacrylamide gel electrophoresis under reducing and non-reducing conditions, it was observed that some of them were dimers whereas others were predominantly monomers [22,35].

A puzzling finding regarding these myotoxins has to do with their enzymatic activity. Two of them (*B. asper* myotoxin I and one of *B. jararacussu* myotoxins) showed phospholipase A_2 activity, whereas the rest of them lacked this effect. [18,22,29,35]

However, all of these myotoxins had biochemical characteristics very similar to those of phospholipases A_2. For instance, *B. nummifer* myotoxin and *B. asper* myotoxin II cross-reacted immunologically with both polyclonal and monoclonal antibodies raised against *B. asper* myotoxin I, a phospholipase A_2[22,35,36,39]. Moreover, the myotoxin from *B. jararacussu,* lacking this enzymatic activity, showed conspicuous homology in the amino acid sequence of the amino terminal region with toxic phospholipases A_2 from crotaline and elapid venoms [29]. Thus, all myotoxins isolated from *Bothrops* venoms may be tentatively classified in the group of ''toxins with phospholipase A_2 structure'' [31] on the basis of their evident biochemical similarities with toxic phospholipases.

Immunochemical data evidenced the presence of components that cross-react with *B. asper* myotoxin I in the venoms of *B. atrox* [18], *B. schlegelli* [37,39] *B. godmani, B. nummifer, B. picadoi* and *Agkistrodon bilineatus* [39]. Moreover, recent unpublished results obtained at the Instituto Clodomiro Picado indicated that there are cross-reacting proteins in the venoms of *B. jararacussu, B. neuwiedi, B. jararaca, B. moojeni, B. colombiensis, B. pictus, B. bilineatus, B. xanthogramma* and *B. brazili.* Therefore, there seems to exist a ''family'' of closely related components in a variety of *Bothrops* venoms having a leading role in the development of muscle tissue damage in these envenomations.

Upon intramuscular injection in mice these myotoxins induced rapid degenerative changes in muscle cells leading to necrosis [18,19,27,29,35]. In the case of *B. asper* and *B. nummifer* myotoxins, the cells underwent early changes characterized by the presence of ''delta lesions'', followed by clumping of myofibrillar material into amorphous, dense masses which alternated with spaces in the cytoplasm devoid of myofilaments [18,19,27]. Afterwards, myofilaments redistributed in the cellular space and necrotic cells became more hyaline and homogeneous in appearance. An inflammatory infiltrate was observed after the sixth hour, reaching maximum levels by 48-72 hr [19,27,29]. Interestingly, after removal of necrotic debris by phagocytes, there was a normal and successful muscle regenerative process, with the formation of myotubes and regenerative muscle fibers [20,29]. The only morphological feature that distinguished these regenerative muscle cells from adult normal muscle cells was the presence of centrally-located nuclei[20,29]. Otherwise, there was no fibrosis nor proliferation of adipose cells substituting necrotic muscle fibers[20,29]. This pattern of regeneration contrasted with the one observed after myonecrosis induced by crude venoms which, by affecting muscle microvasculature, severely hampered the regenerative response [20,21,58].

The mechanism of action has been studied in the cases of *B. asper* myotoxin I and *B. nummifer* myotoxin. The following experimental findings strongly indicate that the first site of action of these toxins is the skeletal muscle plasma membrane: (a) The first morphological lesions detected were focal, wedgeshaped areas of degeneration in the periphery of muscle fibers [18,19,27], very similar to the ''delta lesions'' described in biopsy material

from patients with Duchenne muscular dystrophy [46]. Ultrastructural observations corroborated the fact that the plasma membrane was discontinuous or absent in these early lesions,[19] (b) Both *in vivo* and *in vitro* there was a rapid and drastic release of intracellular markers, such as creatine kinase and creatine, after addition of the toxins *18,22,23,27*, concomitantly with a rapid influx of calcium [18,27] (c) Immunohistochemical findings corroborated the binding of *B. asper* myotoxin I to skeletal muscle plasma membrane[7] (d) *B. nummifer* myotoxin disrupted multilamellar liposomes [27].

Interestingly, although *B. asper* myotoxin I exerted its phospholipolytic activity in muscle tissue[18], it induced the release of creatine kinase and creatine *in vitro* in conditions where phospholipase A_2 activity was inhibited, i.e. when calcium was eliminated and EDTA added to the bathing solution[23]. Similar observations were made with a myotoxic phospholipase fraction from the venom of *B. jararacussu* [62]. These findings pointed towards a dissociation between enzymatic and toxic activities in these toxins, as has been shown for other toxic phospholipases A_2[9,64]. Kini and Iwanaga[32] studied the amino acid sequences of several myotoxic phospholipases and concluded that a variety of myotoxins possess characteristic cationic and hydrophobic domains which are different from the catalytic site. Structural studies suggested that the ''myotoxic segment'' of these molecules is amphiphilic in nature and readily availabe for interaction with membranes [32]. In this regard, it might be relevant the observation that *B. nummifer* myotoxin behaves as an amphiphilic protein when tested by charge-shift electrophoresis [27].

In view of these findings, it is tempting to propose that the myotoxins isolated from *Bothrops* venoms form a group with strong homologies. If indeed myotoxicity depends on a molecular region different from the catalytic site, then the preservation of the catalytic domain is irrelevant from the point of view of myotoxicity. This hypothesis requires support from comparative studies on the amino acid sequences of these ''myotoxins with phospholipase A_2 structure''.

If enzymatic degradation of membrane phospholipids is not the basis of the myotoxic activity of these toxins, an alternative mechanism has to be proposed. On the basis of the findings discussed above, it is possible that they exert their myotoxicity by an interaction of the amphiphilic portion of the molecule with the phospholipid bilayer, inducing membrane leakiness that eventually would lead to irreversible muscle cell injury. The nature of the binding site, as well as the mechanism of membrane disorganization, remain unknown.

How important are these ''myotoxins with phospholipase A_2 structure'' in the total myonecrosis induced by crude *Bothrops* venoms? In the case of *B. asper* venom, where four myotoxin variants have been described[36], Lomonte *et al.*[37,38] observed that incubation of crude venom with horse and rabbit polyclonal antibodies neutralized approximately 75% of the myotoxic effect induced by the venom, a demonstration that this group of toxins are responsible for the major part of muscle damage induced by *B. asper* venom. This conclusion was supported by recent unpublished data in which a monoclonal antibody against myotoxin was able to fully neutralize myotoxicity induced by this venom.

MUSCLE DAMAGE DUE TO ISCHEMIA

Besides myotoxicity caused by the direct action of toxins on muscle cells, there is also muscle damage due to ischemia in bothropic envenomations[19,57]. Although there are no studies describing biochemical evidences of ischemia in muscle affected by venoms, it is likely that there is ischemia due to (a) drastic damage to the microvasculature leading to hemorrhage, and (b) alterations in larger vessels, particularly in intramuscular arteries.

Damage to the capillary network is due to the action of hemorrhagic components (see below). As a consequence of their action, capillaries collapse and blood leaks to the extravascular compartment[48]. This phenomenon causes an immediate effect on the blood supply to muscle cells, inducing ischemia. In this regard, it is relevant to point out that experimental inoculations of hemorrhagic components from the venom of *B. jararaca* resulted in immediate hemorrhage, followed by widespread muscle damage[59]. Queiroz *et al.*[59] suggested that damage to muscle fibers after injection of hemorrhagic toxins might be a consequence of the ischemia which developed after the drastic hemorrhagic effect. However, it is also possible that some hemorrhagic components exerted a direct myotoxic activity, as has been proposed for viriditoxin and hemorrhagic toxin *b*, from the venoms of *Crotalus viridis*[10,13] and *Crotalus atrox*[53] respectively. Studies on the action of hemorrhagic toxins on skeletal muscle *in vitro* are required in order to discriminate between direct myotoxicity and myotoxicity due to ischemia. Evidently, there is an urgent need of pathological studies with purified hemorrhagic components from *Bothrops* venoms.

Ischemic muscle damage can also occur as a consequence of alterations in intramuscular arteries. Homma and Tu[28] described the occurrence of arterial lesions in mice injected intramuscularly with the venoms of *B. atrox, B. nasuta, B. nummifer, B. picadoi* and *B. schlegelii*. These lesions included disintegration of endothelial cells, leucocytic infiltration beneath the endothelium, necrosis and disappearance of the smooth muscle fibers in the media, hemorrhage and insudation of a fibrin-like substance into the subendothelial and medial layers, and formation of mural thrombi. Queiroz and Petta[57] described hyaline necrosis of the media in arteries of muscle tissue obtained from mice injected with *B. alternatus* venom. Moreover, inoculation of high doses of *B. jararacussu* venom induced hyalinization of the media of some arteries, as well as loss of endothelium[58]. Little effort has been made on the isolation and characterization of factors affecting large vessels. However, it is interesting that the hemorrhagic component HF_2 of *B. jararaca* venom induced necrosis of intramuscular arteries [59]. Arterial necrosis and thrombosis may have dramatic effects in the blood supply to muscle tissue, originating ischemia in groups of muscle cells. This, in turn, might induce irreversible cell damage, also affecting the process of muscle regeneration[57].

An additional element which may impair the blood supply to muscle tissue is the elevation in the interstitial pressure of some muscle compartments, due to the accumulation of large volumes of fluid after the action of hemorrhagic and edemaforming toxins on the microvasculature. Although this phenomenon has received little attention at the experimental level, so-

me studies suggested that it may be a relevant component in the pathogenesis of local tissue damage in crotaline envenomations[12].

HEMORRHAGE INDUCED BY *BOTHROPS* VENOMS

Hemorrhage is one of the most characteristic effects induced by crotaline venoms (see reviews by Ohsaka[48] and Ownby[50]). The pioneer work made by Ohsaka and his group on the nature and mechanism of action of hemorrhagic components purified from the venom of *Trimeresurus flavoviriridis*, a close relative of *Bothrops*[6], led to the proposal that these toxins induce hemorrhage by ''diapedesis'', i.e. by opening the intercellular junctions between endothelial cells in capillaries, with the consequent extravasation[48]. In contrast, Ownby *et al.*[52,53] presented strong evidence indicating that several hemorrhagic toxins from the venoms of *Crotalus atrox* and *Crotalus horridus* induce hemorrhage ''per rhexis'', i.e. by disrupting the integrity of endothelial cells, escaping the erythrocytes through gaps formed within these cells.

Many hemorrhagic components isolated from crotaline venoms are proteases[67], and this enzymatic activity may be relevant to the process of capillary damage. Several hemorrhagic toxins degrade collagen, and it is known that collagen type IV is a component of the basal lamina that surrounds endothelial cells in the capillaries[66] Ohsaka *et al.*[49] demonstrated that several hemorrhagic components from *Trimeresurus flavoviridis* venom released proteins and carbohydrates from isolated glomerular basement membrane. Thus, it might be that hemorrhagic toxins induce damage to the microvasculature by first affecting the integrity of the basal lamina, inducing the collapse of the capillary structure. However, a direct cytotoxic effect on endothelial cells cannot be ruled out at the present time. Further work is required to elucidate the mechanism of action of hemorrhagic toxins.

Five hemorrhagic toxins have been isolated and characterized from *Bothrops* venoms: HF_1, HF_2 and HF_3 from *B. jararaca*[41,43] and NHF_a and NHF_b from the venom of *B. neuwiedi*[42,43]. These five toxins have similar molecular weights (46,000-62,000), being all of them proteins with acidic pI. They were described as heat labile metalloproteins, inhibited by EDTA, EGTA and 1.10 phenanthroline[41,42,43]. Interestingly, many hemorrhagic components isolated from snake venoms were Zn^{2+} — containing proteases which lost their activity upon Zn^{2+} removal[4,67].

The hemorrhagic toxins isolated from *Bothrops* venoms displayed proteolytic activity on casein, with the exception of HF_3 from *B. jararaca*[43]. However, all of them hydrolyzed fibrinogen and the B-chain of oxidized insulin[43]. In this regard, there has been a controversy concerning the proteolytic activity of hemorrhagic toxins[4]. Since casein is not the best substrate for this enzyme, it is necessary to test this activity on other substrates such as dimethylcasein and collagen. It is likely that the large majority of hemorrhagic toxins are proteases with limited substrate specificity.

Besides these five bothropic hemorrhagic components, two additional proteases, bothropasin from *B. jararaca* venom and moojeni protease A from *B. moojeni* venom, induced hemorrhage, although at much higher doses[40,59]. These enzymes were highly active in terms of proteolysis, having a low hemorrhagic effect. Immunologically, these two proteases clearly

differed from hemorrhagic components[40]. On the other hand, polyclonal antibodies raised against hemorrhagic factors reacted with several *Bothrops* venoms, indicating that immunologically-related proteins are present in a variety of these venoms.

EDEMA AND INFLAMMATION

Bothropic envenomations are also characterized by the rapid development of edema and inflammation at the site of venom injection.[2,5,65]. Despite its clinical relevance, the study of venom-induced edema has received little attention, as demonstrated by the scarcity of reports on the isolation and characterization of edema-forming toxins.

When tested on the foot-pad assay described by Yamakawa *et al.*[70], the venom of *B. asper* induced a rapid edema which remained at a high level even at 24 hours[16]. The edema induced by Bothrops snake venoms is problably due to the action of a variety of substances: (a) Hemorrhagic toxins which disrupt the microvasculature inducing extravasation[48,50]. (b) Toxins acting directly on the endothelial cells of the capillaries and venules, increasing their permeability[48,50]. (c) Venom components (phospholipases or cytotoxins) which induce the release of histamine from mast cells. (d) Phospholipases A_2 that release arachidonic acid from phospholipids in cell membranes, initiating the pathway leading to the synthesis of prostaglandins[60] (e) Proteases that act on plasma kininogens, liberating kinins (e.g. bradykinin), as was demonstrated by Rocha e Silva *et al.*[61] back in 1949. Moreover, kallikrein can be activated by factor XII of the coagulation cascade, once this factor is activated after damage to the vasculature. (f) components of the complement cascade, particularly C3a and C5a, which participate in the inflammatory reaction[60]. Due to the presence of many pharmacologically active proteins in *Bothrops* venoms, it is likely that the development of edema is due to the combination of these elements.

Lomonte[34], Gutiérrez *et al.*[25] and Rojas *et al.*[63] observed that neutralization of edema-forming activity of Central American crotaline venoms by a polyvalent antivenom is difficult to achieve. Due to the relevance of edema in bothropic envenomations, it is necessary to identify and characterize edema-forming components in these venoms, as well as to study their mechanism of action, in order to design new therapeutic strategies to confront this significant local effect.

Besides edema, there is a conspicuous cellular component in the inflammatory reaction in bothropic envenomations. In the case of intramuscular injection of *B. asper* venom, inflammatory infiltrate started after the sixth hour and reached its highest level by 48-72 hours[24]. Initially, the predominant cell type was the polymorphonuclear neutrophil but, at later time periods, macrophages became more abundant.[24] It was proposed that degradation of myofibrillar proteins in necrotic muscle cells after injection of *B. asper* venom was accomplished by proteases from invading phagocytic cells.

FINAL REMARKS: THE NEED OF AN INTEGRATED VIEW

Muscle tissue, where local effects take place, is complex both anatomically and functionally. *Bothrops* venoms affect not only skeletal muscle cells,

but also intramuscular arteries, microvessels and nerves. Therefore, the study of local effects must include an integrated analysis of the effect of these venoms on the various cell types and extracellular structures forming a given muscle.

Until now there have been few studies in this direction. For instance, it is not known how relevant is the ischemia in the development of muscle damage. Furthermore, it is necessary to determine which phenomenon is more important in the development of ischemia, if damage to microvasculature by hemorrhagic toxins or lesions in intramuscular arteries. This particular point is especially relevant, since the design of new therapeutic avenues will depend on this information. For instance, if ischemia is basically due to microvessel damage, then there is a good point in using angiogenic substances to stimulate revascularization[11] which, in turn, would contribute to a more successful skeletal muscle regeneration. If, on the other hand, arterial damage predominates, the approach must be different.

Two additional points that need to be investigated are: (a) The role of axonal degeneration in local tissue damage, particularly in regard to the process of muscle regeneration; and (b) the effect of *Bothrops* venoms on the extracellular matrix, including the basal lamina surrounding muscle fibers and capillaries. Since this matrix is determinant in the maintenance of an adequate spatial relationship between cells, its alteration may have drastic effects in the muscle as a whole.

In conclusion, it is necessary to isolate and characterize additional components from *Bothrops* venoms which induce myonecrosis, hemorrhage and edema. It is also important to study the immunological relationships of these components, in order to have a more rational basis for selecting the venoms to be used in the production of antivenoms. Concomitantly, the integration of biochemical, physiological and pathological studies is essential, in order to gain a comprehensive knowledge of these complex and challenging phenomena.

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RESUMO: A revisão focaliza os efeitos locais induzidos pelo veneno de *Bothrops* caracterizados por mionecrose, hemorragia e edema, de evolução rápida. As miotoxinas classificadas como fosfolipase A_2 pela estrutura, embora algumas não possuam essa atividade enzimática; as hemorraginas caracterizadas como metaloproteínas acídicas, e os fatores edematogênicos, estão presentes nos venenos botrópicos. Várias miotoxinas e hemorraginas foram purificadas de venenos botrópicos.

UNITERMOS: Venenos de *Bothrops*, miotoxinas, mionecrose, hemorragia, edema.

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ISOLATION AND PROPERTIES OF A PHOSPHOLIPASE A2 FROM THE VENOM OF THE SNAKE *BOTHROPS MOOJENI* (CAISSACA)

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ABSTRACT: BM-PLA$_2$, a phospholipase A2, was purified from the venom of the snake *Bothrops moojeni* by chromatography on Sephadex G-100, DEAE-Sephadex A-50 and rechromatography on DEAE-Sephadex A-50. BM-PLA$_2$ is an acidic protein of pI 4.6. The enzyme is homogeneous in polyacrylamide gel electrophoresis and ultracentrifugal analyses. The $s^o_{20,w}$ and $D^o_{20,w}$ are 2.08 S and 14.9 $\times$ 10^{-7} cm^2/sec., respectively. The molecular weight of 11,300 was calculated by s^o/D^o ratio. In SDS-polyacrylamide gel electrophoresis the denatured and reduced enzyme exhibits a single polypeptide chain of molecular weight of 11,900. The enzyme is cross-linked by four disulphide bridges and has histidine as N-terminal amino acid. Chemical modifications of BM-PLA$_2$ with p-bromophenacylbromide and N-bromosuccinimide cause complete loss of enzymatic activity. The purified phospholipase A2 is nontoxic, nonhemorrhagic and no edema forming.
KEY WORDS: Venom, *Bothrops moojeni*, phospholipase A2.

INTRODUCTION

Snake, bee and scorpion venoms and mammalian pancreas are rich sources of phospholipase A2. Snake venoms contain several phospholipase A2 enzymes which differ in enzymatic and pharmacological features. A great number of phospholipases A2 have already been purified from the venoms of snakes of the Elapidae and Viperidae families. However very little is known about phospholipases A2 from venoms of *Bothrops* species. The venoms of *Bothrops* species were considered by Marinetti[23] and Mebs[24] as poor sources of phospholipase A2. Vidal and Stoppani[34] and Vidal *et al*[33], on

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the contrary, observed a high phospholipase A2 activity in various venoms of *Bothrops* species, such as *Bothrops jararaca, Bothrops jararacussu* and *Bothrops atrox.* Until now phospholipases A2 of venoms of *Bothrops* species were isolated only from *B. neuwiedi* (Vidal and Stoppani[34]), *B. asper* (Alagon *et al*[1]; Gutierrez *et al*[11]) and *B. alternatus* (Nisenbon *et al*[25]).

In the venom of *Bothrops moojeni* was found a high phospholipase A2 activity associated to high blood-clotting, high proteolytic and low hemorrhagic activities. From this venom was isolated the coagulant enzyme batroxobin (Stocker and Barlow[30]). Also the major proteolytic enzyme, *moojeni* protease A, was already isolated (Assakura *et al*[2]).

In the present study, a phospholipase A2 from the venom of *Bothrops moojeni* was isolated to homogeneity and some biochemical, biophysical and physiopathological properties were characterized.

MATERIALS AND METHODS

Purification of phospholipase A2

Dried crude venom (1.8g) of *Bothrops moojeni* was dissolved in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.5 mM $CaCl_2$ and 0.02% sodium azide (buffer A) and clarified by centrifugation. The supernatant solution containing 1.62g of protein in 12.5 ml was chromatographed on a column (2.6 × 80 cm) of Sephadex G-100 previously equilibrated in buffer A under the conditions described by Assakura *et al*[2] for the isolation of *moojeni* protease A. The fractions from peak II containing the phospholipase A2 activity were combined and lyophilized. A solution of 530 mg of protein in 17 ml, dialyzed against buffer A, was applied to a 2.5 × 50 cm column of DEAE-Sephadex A-50 equilibrated with buffer A. After washing the non adsorbed protein, a linear gradient up to 0.3 M NaCl in the same buffer (mixing vessels 500 ml) was applied. The fractions in the protein peak, which contained the phospholipase A2 activity, were combined, lyophilized and dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) containing 0.5 mM $CaCl_2$, 0.02% sodium azide and 0.075 M NaCl (buffer B), and applied to a 2.5 × 50 cm column of DEAE-Sephadex A-50 previously equilibrated with buffer B. The fractions (45 to 60) with constant specific activity were combined and lyophilized.

Phospholipase A2 activity

This was determined using egg-yolk lecithin as substrate according to Kornalik and Master[17]. Lysolecithin released as a result of enzyme activity was assayed by the hemolytic effect on washed horse red blood cells. One unit of phospholipase A2 activity was defined as the amount of protein which causes 50% hemolysis corresponding to an increase in O.D. of 1.0 at 540 nm. Specific activity is expressed as Units per mg of protein.

Caseinolytic activity

Activity was determined by a modification of the method of Kunitz[18], as described by Mandelbaum *et al*[22]. One unit of caseinase activity is defined as the amount of enzyme yielding an increase in O.D. of 1.0 per min at 750 nm.

Blood-clotting activity

This activity was assayed on citrated horse plasma [1 ml of trisodium citrate 3.8% (w/v) for 10 ml of blood] and measured as described by Henriques *et al*[13].

Hemorrhagic activity

The hemorrhagic activity was assayed by intradermal injection into the backs of mice with doses varying from 2 to 20 μg of venom or isolated phospholipase A2 protein in a volume of 0.1 ml. Four Swiss mice (20-22g) were used for each dose. The mice were sacrificed 2 h later by ether inhalation, the skins removed and the hemorrhagic lesions were evaluated on the visceral side and also on the dorsal surface of the musculature.

Estimation of protein

Protein concentration was measured by the method of Lowry *et al*[20] using bovine serum albumin as standard, or by spectrophotometric determination of absorption at 280 nm.

Polyacrylamide gel electrophoresis

Polyacrylamide 10% gel electrophoresis in Tris-glycine buffer, pH 8.5 was performed according to the method of Maizel[21] with a 2.5% gel spacer following the method of Davis[4]. Runs were of 3 h with 1.25-1.50 mA per tube (0.5 × 8.0 cm), bromophenol blue being used as indicator. Gels were stained for 16 h at room temperature with 0.05% Coomassie brilliant blue R in 12.5% trichloroacetic acid, containing 40% methanol, and destained with several changes of 7.5% acetic acid -5% methanol. For glycoprotein detection the gels were stained with periodic acid-Schiff according to the procedure of Zacharius *et al*[40] as modified by Glossmann and Neville[9].

Molecular weight determination

Sodium dodecyl sulfate (SDS) 10% (w/v)-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn[36]. Gels were cast in 0.5 cm i.d. tubes to a height of 8 cm, and the protein sample contained 1% SDS (w/v) and 1% 2-mercaptoethanol (v/v) in 0.01 M phosphate buffer (pH 7.4). The mobilities of ovalbumin, bovine serum albumin, ribonuclease and γ -globulin (H-and L-chains) were measured in comparison to that of bromophenol blue.

Analytical isoelectric focusing

Isoelectric focusing was conducted in a Pharmacia Flat Bed Apparatus FBE 3000 in a layer of 5% polyacrylamide gel with 2% Ampholine (LKB) in the pH range 3-6, at 200 V for 20 h and 4°C, according to the procedure of Vesterberg[32]. Ovalbumin (pI 4.6) and methyl blue (pI 3.6) were run as markers. The pH gradient in the layer was measured potenciometrically after 4 h diffusion of equal samples cut from the gel in 1 ml of water at 25°C.

Amino acid analyses

Amino acid analyses were carried out with a Technicon amino acid analyzer (Spackman *et al*[28]; Hamilton[12]). Lyophilized salt-free samples were hydrolyzed in 3N p-toluene sulfonic acid containing 0.2% 3(2-aminoethyl) indol at 110°C for 24,48 and 72 h according to the method of Liu and Chang[19]. Half-cystine content was determined as cysteic acid after performic acid oxidation (Hirs[14]) prior to hydrolysis for 16 and 24 h in constant

boiling HCl (6N) at 110°C. Tryptophan and tyrosine were also determined spectrophotometrically in 0.1N NaOH, as described by Goodwin and Morton[8].

Partial specific volume

$\bar{V}$ was calculated from the amino acid composition of the enzyme, according to the procedure of Cohn and Edsall[3].

Ultracentrifugal analyses

These analyses were performed in a Spinco Model E ultracentrifuge equipped with RTIC control unit and phase plate. Both Schlieren and interference optics were employed. All measurements of ultracentrifuge patterns were made with a Zeiss small toolmaker's measuring microscope. Sedimentation and diffusion coefficients were determined at 20°C in 0.1 M Tris-HCl containing 0.1 M NaCl and 2 mM $CaCl_2$ buffer solution (pH 7.6), in double sector, capillary type, synthetic boundary cells. Sample concentrations were in the range of 0.9 to 3 mg per ml. Sedimentation velocity experiments were performed with Schlieren optics at 56,000 revs/min (D rotor). The sedimentation coefficients were calculated according to the method of Schachman[27]. Diffusion coefficients were determined at 6,000 revs/min (J rotor), and the pictures analysed by the height-area method of Ehrenberg[6]. Sedimentation and diffusion coefficients were corrected to standard conditions of density and viscosity of water at 20°C. Low speed sedimentation equilibrium experiments were performed at 20°C with double sector cells having a 12mm light path, fitted with quartz windows. The column heights of enzyme solution were of 3 mm (Van Holde and Baldwin[31]; Yphantis[39]). Fluorcarbon FC-43 was used as base fluid to give a transparent cell bottom. The rotor speeds were sufficiently low for the solute concentration at the meniscus not to be zero. Equilibrium was established when no measurable change occurred in fringe displacement with time. Molecular weights were determined with samples of 1.8 and 0.9 mg per ml.

Oxidation with N-bromosuccinimide (NBS)

The modification of the phospholipase A2 was perfomed essentially according to the procedure of Spande and Witkop[29]. The enzyme sample (0.72 mg which corresponds to O.D. at 280 nm of 1.0) was dissolved in 1 ml of 0.1 M acetate buffer, pH 4.0. To this solution 5 mM NBS were added in 10 μl portions. After each addition the change in absorbance at 280 nm was measured and the number of oxidized tryptophan residues was estimated from the decrease in the absorbance at 280 nm.

Modification with p-bromophenacylbromide

The enzyme (0.5 mg) was dissolved in 0.5 ml of 0.025 M Tris-HCl buffer, pH 7.5 containing 0.2 mM EDTA. To this solution 25 μl of 2 mM p-bromophenacylbromide were added. After 4 and 6 h at room temperature, phospholipase A2 activity was assayed in aliquots of the reaction mixture, as described above.

Free sulphydryl group

Free sulphydryl group was measured according to the method of Ellman[7]. The reactions were performed with 0.025 to 0.05 μmoles of the enzyme in 0.1 M phosphate buffer, pH 8.0 in presence of 1% SDS and 10^{-4}

M EDTA. The absorbance at 412 nm was measured 30 min and 1 h after addition of 5,5'-dithiobis (2-nitrobenzoic acid).

N-terminal amino acid analysis

This was carried out using dansyl chloride as described by Gros and Labouesse[10] with denatured and reduced enzyme according to the procedure of Weber and Osborn[36]. The DNS-amino acids obtained after acid hydrolysis were identified by the method of Woods and Wang[37].

RESULTS

Isolation of phospholipase A2

A significant phospholipase A2 activity was observed in the venom of *Bothrops moojeni* when it was fractionated by the procedure used for the isolation of *moojeni* protease A (Assakura *et al*[2]). The phospholipase A2 activity was found in the second peak of the gel filtration on Sephadex G-100. This fraction contained also various proteolytic enzymes, being however free from the blood-clotting and hemorrhagic activities present in the venom

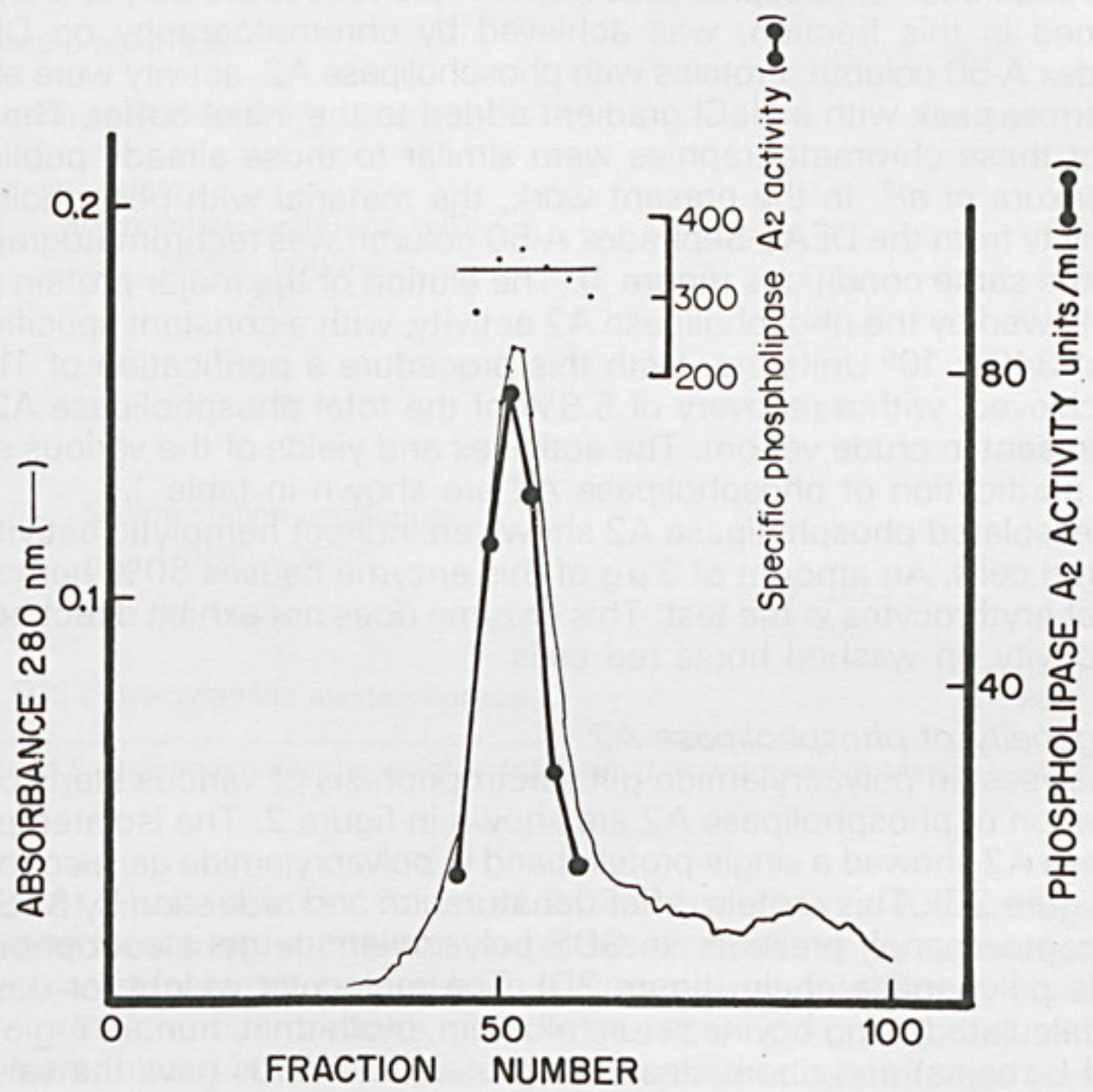

FIG. 1. ELUTION PROFILE OF BM-PLA$_2$ FROM RECHROMATOGRAPHY OF DEAE-SEPHADEX A-50.
A sample of phospholipase A2 of 22 mg in 7 ml, previously dialyzed against 0.05 M Tris-HCl — 0.5 mM $CaCl_2$ buffer, pH 7.5 containing 0.02% sodium azide and 0.075 M NaCl was applied to a DEAE-Sephadex A-50 column (2.5 x 50 cm) previously equilibrated with the same buffer solution.

TABLE 1

YIELD AND ACTIVITY OF PHOSPHOLIPASE A2 ISOLATED FROM THE VENOM OF ***BOTHROPS MOOJENI.***

Purification steps	Protein		Phospholipase A2 activity			
	mg	%	specific 10^{-6}x Units/mg	total x10^{-6}	%	purification factor
Crude venom	1620	100	31.2	50544	100	1.0
Sephadex G-100	530	32.8	43.5	23098	46	1.4
DEAE-Sephadex A-50 (first chromatography)	22	1.37	185	4096	8.1	5.9
DEAE-Sephadex A-50 (rechromatography)	8.7	0.54	340	2958	5.8	11

Specific activity is expressed as described in "Materials and Methods"

A good separation of phospholipase A2, from the various proteolytic enzymes contained in this fraction, was achieved by chromatography on DEAE-Sephadex A-50 column. Proteins with phospholipase A2 activity were eluted in a narrow peak with a NaCl gradient added to the initial buffer. The patterns of these chromatographies were similar to those already published by Assakura *et al*[2]. In the present work, the material with phospholipase A2 activity from the DEAE-Sephadex A-50 column was rechromatographed under the same conditions (figure 1). The elution of the major protein peak was followed by the phospholipase A2 activity, with a constant specific activity of 340 x 10^6 Units/mg. With this procedure a purification of 11-fold was achieved, with a recovery of 5.8% of the total phospholipase A2 activity present in crude venom. The activities and yields of the various steps of the purification of phospholipase A2 are shown in table 1.

The isolated phospholipase A2 shows an indirect hemolytic activity on horse red cells. An amount of 3 μg of this enzyme causes 50% hemolysis of intact erythrocytes in the test. This enzyme does not exhibit direct hemolytic activity on washed horse red cells.

Homogeneity of phospholipase A2

Analyses in polyacrylamide gel electrophoresis of various steps of the purification of phospholipase A2 are shown in figure 2. The isolated phospholipase A2 showed a single protein band in polyacrylamide gel electrophoresis (figure 2C). This protein, after denaturation and reduction by SDS and 2-mercaptoethanol, presents in SDS-polyacrylamide gel electrophoresis, a single polypeptide chain (figure 2D). The molecular weight for this enzyme calculated using bovine serum albumin, ovalbumin, human γ-globulin (H and L-chains) and ribonuclease as protein standards gave the value of 11,900.

Analytical ultracentrifugation using sedimentation velocity experiments yielded symmetrical single schlieren patterns. The values of the sedimentation and diffusion coefficients varied with the protein concentration. When concentration of phospholipase A2 varied from 3.6 to 0.9 mg/ml, the values for $s_{20,w}$ varied from 2.99 to 2.53, while $D_{20,w} \times 10^7$ varied from 12.55 to

TABLE 2

BIOPHYSICAL PROPERTIES OF *BOTHROPS MOOJENI* PHOSPHOLIPASE A2 (BM-PLA_2)

Parameter		Value
Sedimentation coefficient ($s_{20,w}$, S)		
protein (mg/ml)		
3.6	2.99	
1.8	2.99	
1.5	2.85	
0.9	2.53	
		2.08 ($s^o_{20,w}$)
Diffusion coefficient ($D_{20,w}x10^7$, cm^2/sec)		
protein (mg/ml)		
3.6	12.55	
1.8	12.70	
1.5	13.99	
0.9	14.21	
		14.90 ($D^o_{20,w}x10^7$)
Partial specific volume ($\bar{v}$, ml/g)		0.704
Isoelectric point (pI)		4.60
Extinction coefficient ($E^{1\%}_{1cm}$, 280 nm)		13.96
Molecular weight		
Sedimentation-diffusion (s/D)		
protein (mg/ml)		
3.6	19,600	
1.8	19,380	
1.5	16,800	
0.9	14,650	
		11,300 (s^o/D^o)
Sedimentation equilibrium		
protein (mg/ml)		
1.8	19,100	
0.9	13,900	
SDS-polyacrylamide electrophoresis		11,900

Values of s, D, $\bar{v}$ and molecular weights determined as described in Materials and Methods.

14.21. At infinite dilution, values of 2.08 S and 14.90 x 10^{-7} cm^2/sec were obtained for the sedimentation and diffusion coefficients, respectively. The molecular weights determined from sedimentation and diffusion values, with these protein concentrations, gave values varying from 19,600 to 14,600. The extrapolated value of 11,300 was obtained at zero protein concentration. Molecular weights were also calculated from sedimentation equilibrium experiments. By this method the molecular weights of phospholipase A2 in the concentrations of 1.8 and 0.9 mg/ml, gave values of 19,000 and 13,900, respectively.

By isoelectric focusing in thin layer of polyacrylamide gel, phospholipase A2 showed to be an acidic protein with a pI of 4.60.

This homogeneous phospholipase A2 isolated from *B. moojeni* venom, was named BM-PLA_2. The biophysical constants of BM-PLA_2 are shown in table 2.

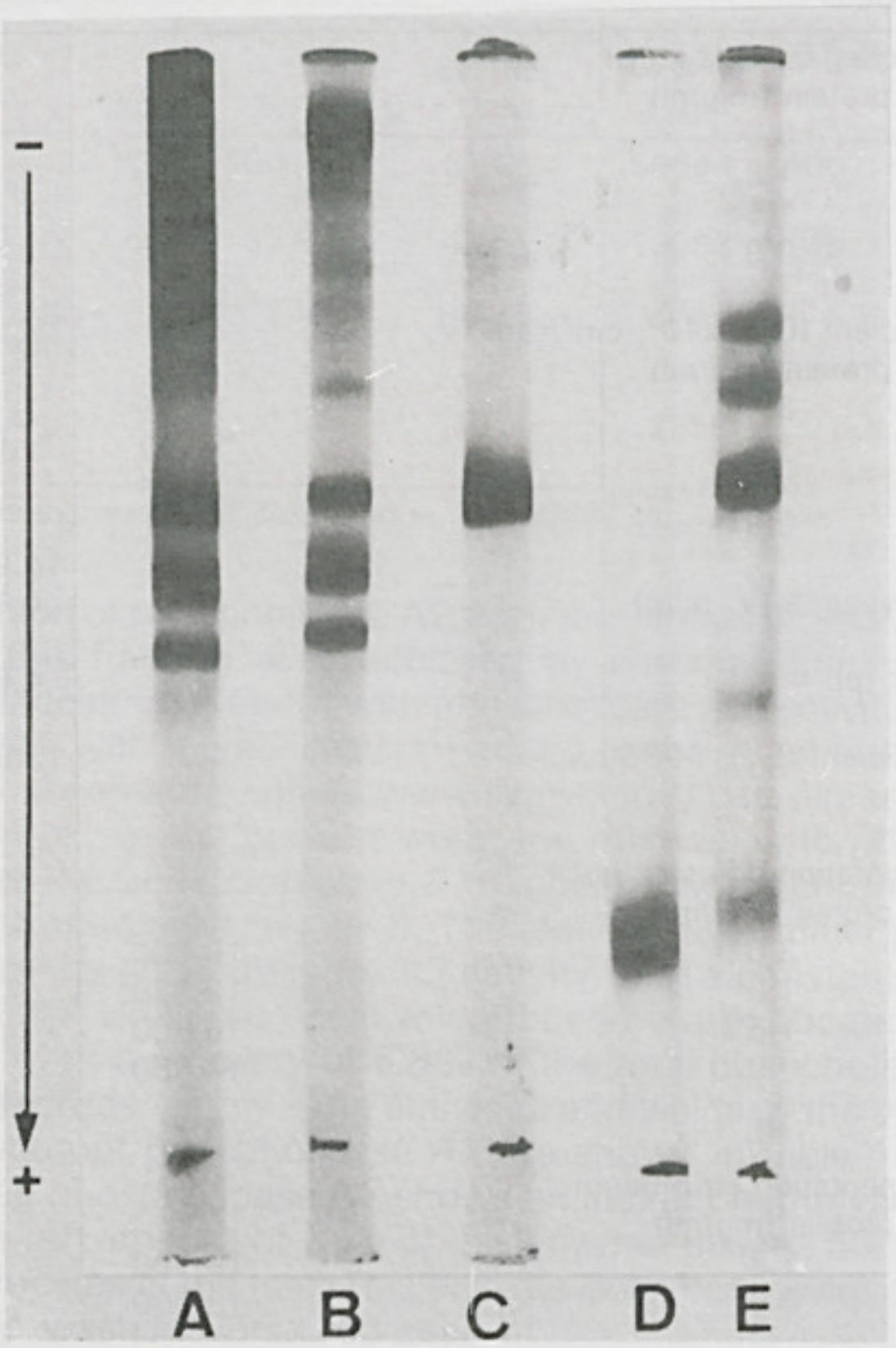

FIG. 2. POLYACRYLAMIDE GEL ELECTROPHORESIS.
(A) Crude venom (80 μg). (B) Proteins from peak II of Sephadex G-100 column (30 μg). (C) Isolated *B. moojeni* phospholipase A2 (15 μg). (D) reduced enzyme (12 μg). (E) mixture of reduced bovine serum albumin, ovalbumin, γ-globulin (H and L-chains) and ribonuclease used as standards for molecular weight measures.

Amino acid composition

The amino acid composition of BM-PLA_2 is shown in table 3. The protein has a high content of acidic amino acids, 11 Asp and 8 Glu residues. BM-PLA_2 presents also high contents of 1/2 Cys, Gly and Lys. Tryptophan and tyrosine were also determined spectrophotometrically. The number of residues given by this procedure were the same as those obtained by hydrolysis with p-toluene sulfonic acid. The amino acid analyses showed that BM-PLA_2 contained 8 cysteine residues. Free sulfhydryl groups were not found. The method of Ellman[7] indicated less than 0.1 residues per mole of pro-

TABLE 3

AMINO ACID COMPOSITION OF *BOTHROPS MOOJENI* PHOSPHOLIPASE A2 ($BM\text{-}PLA_2$). HYDROLYSIS WITH 3 N p-TOLUENE SULFONIC ACID.

Amino Acid	Residues/mole of enzyme	nearest integer
ASP	11.32	11
THR[a]	3.98	4
SER[a]	4.75	5
GLU	7.91	8
PRO	3.74	4
GLY	7.64	8
ALA	3.62	4
VAL[b]	3.96	4
1/2CYS[c]	7.76	8
MET	1.15	1
ILEU[b]	2.45	2
LEU	2.12	2
TYR	4.00	4
PHE	4.38	4
LYS	5.80	6
HIS	1.68	2
TRP	1.01	1
ARG	3.61	4
NH_3[a]	12.21	7
TOTAL		89

Average values of duplicate analyses of 24,48 and 72 h acid hydrolysis. Number of residues based on molecular weight of 11,900.
a — extrapolated to zero time hydrolysis.
b — values of 72 h hydrolysis.
c — determined as cysteic acid after perfomic acid oxidation.

tein. Based on amino acid composition, the minimum molecular weight of $BM\text{-}PLA_2$ corresponds to 9,400. This value is 20% lower than those estimated by ultracentrifugal analyses and SDS-polyacrylamide gel electrophoresis. Considering that this low value given by the amino acid composition could be due to the presence of carbohydrates, $BM\text{-}PLA_2$ was assayed for glycoprotein. However, no Schiff's stain was observed.

The N-terminal amino acid of $BM\text{-}PLA_2$ was found to be histidine.

Stability of $BM\text{-}PLA_2$ at pH variation

The effect of pH on the enzyme was tested with solutions adjusted with dilute NAOH or HCl to various pHs. After 6 h at room temperature, the phospholipase A2 activity was measured. The enzyme is stable, as no significant alteration in enzymatic activity was observed at pHs ranging from 4 to 8. However, an increase of 30% of its activity was observed when the enzyme was submitted to pHs 8 to 10 and loss of 25% of the activity was observed at pHs below 4.

Chemical modifications

$BM\text{-}PLA_2$ lost completely its activity after oxidation with N-bromosuccinimide. The tryptophan residue was modified without denatu-

ration of BM-PLA_2 with urea. The modified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis, with the same mobility as the native enzyme, excluding the possibility of a split in the polypeptide backbone. Treatment for 4 h of BM-PLA_2 with p-bromophenacylbromide resulted also in total loss of activity. Only one of the two histidine residues of the enzyme was alkylated. The other histidine was not modified and was found as N-terminal amino acid.

Biological activities

Some biological tests were assayed with the isolated BM-PLA_2 in mice. BM-PLA_2 was not toxic when injected intraperitoneally. No deaths after 24 h were observed in the injected mice with various doses up to 1 μg/g body weight (a minimum of 6 animals were used per dose). However crude venom in dose of 7.5 μg/g body weight, was enough to kill mice at the end of 3 h. Also no edema was observed when injected in the foot pads of the hind limbs with doses up to 25 μg of BM-PLA_2 following the method of Yamakawa *et al*[38]. Hemorrhage was also not detected even with doses of 20-25 μg of BM-PLA_2 injected intradermally, intraperitoneally or into the foot pads. With crude venom hemorrhage and edema were already observed with doses of 2 μg. This shows that BM-PLA_2 possesses no lethal, hemorrhagic or edema-forming activities.

DISCUSSION

The isolated BM-PLA_2 showed to be homogeneous by polyacrylamide gel electrophoresis, SDS-polyacrylamide gel electrophoresis, isoelectrofocusing, ultracentrifugation and N-terminal amino acid analysis.

BM-PLA_2 in ultracentrifugal analyses showed that both sedimentation and diffusion coefficients varied with the protein concentration. The decreasing values of the molecular weight calculated by s/D ratio or sedimentation equilibrium with the decreasing of the protein concentration indicate that BM-PLA_2 is found in reversible associated form. The extrapolated value of 11,300 daltons at zero protein concentration for the molecular weight of BM-PLA_2 is close to the molecular weight of 11,900 determined for the single polypeptide chain in SDS-polyacrylamide gel electrophoresis. Concentration-dependent aggregation was also observed in phospholipases A2 from the venoms of *Agkistrodon halys blomhoffii* (Kawauchi *et al*[16]) and of *Naja naja naja* (Deems and Dennis[5]).

BM-PLA_2 is an acidic protein of pI 4.6, with predominance of Asp and Glu residues over the basic amino acids. BM-PLA_2 contains 8 half-cystine residues, a lower content when compared to the phospholipases A2 isolated from elapidic venoms, which contain, in general 12 to 14 residues. Our results are close to the half-cystine content of phospholipases P_1 and P_2 isolated from the venom of *B. asper* (Alagon *et al*[1]). Compared to the phospholipases A2 of the elapidic venoms, the low content of half-cystine residues of phospholipases A2 of venoms of *Bothrops* species suggests a different cross-linking.

The loss of activity by chemical modification of BM-PLA_2 by N-bromosuccinimide is correlated to the destruction of the single tryptophan residue of the enzyme molecule. The oxidation of this tryptophan without

prior denaturation of the enzyme suggests that this amino acid must be on the surface of the native enzyme. Modification of the histidine residues of BM-PLA_2 with p-bromophenacylbromide also caused complete loss of enzyme activity. Volwerk *et al*[35] demonstrated that the histidine residue located at the active site of pancreatic phospholipase A2 is modified by p-bromophenacylbromide. Considering that one histidine of the modified BM-PLA_2 was found as the N-terminal amino acid we concluded that the histidine essential for the catalytic activity was alkylated by p-bromophenacylbromide.

The acidic phospholipase A2 isolated in this study, although having a high indirect hemolytic activity, is nonhemorrhagic, nontoxic and no edema forming.

Acid phospholipases A2 from snake venoms are usually less toxic in contrast to the basic ones (Rosenberg[26]). In spite of this fact, a phospholipase A2 with lethal activity was isolated from the venom of *Bothrops alternatus* (Nisenbon *et al*)[25]. On the other hand, not all basic phospholipases A2 are highly toxic. One of the phospholipases A2, VRV PL-VIIIa, isolated from the venom of *Vipera russelli*, with a low indirect hemolytic activity, is less toxic compared to the whole venom (Kanturi and Gowda[15]).

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RESUMO: Do veneno de *Bothrops moojeni* foi isolada uma fosfolipase A2, BM-PLA_2, por cromatografia em Sephadex G-100, DEAE-Sephadex A-50 e recromatografia em DEAE-Sephadex A-50. BM-PLA_2 é uma proteína ácida de pI 4,6. A enzima mostrou ser homogenea por eletroforese em gel de poliacrilamida e análises de ultracentrifugação. Os valores para $s°_{20,w}$ e $D°_{20,w}$ são de 2,08 S e 14,90 x 10^{-7} cm^2/ seg, respectivamente. O peso molecular calculado da relação $s°/D°$ é de 11.300 daltons. A enzima desnaturada e reduzida apresenta, por eletroforese em SDS-gel de poliacrilamida, uma única banda de peso molecular 11.900. BM-PLA_2 possui quatro pontes dissulfeto e seu N-terminal é histidina. Modificações químicas da enzima com p-bromofenacilbrometo e N-bromosuccinimida resultaram na perda total da atividade enzimática. A fosfolipase A2 isolada não causa hemorragia, não é tóxica e não é edematogênica.
UNITERMOS: Veneno, *Bothrops moojeni*, fosfolipase A2.

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51(4):239-254, 1989.

COLETÂNEA DE RESUMOS DE TRABALHOS PUBLICADOS PELOS PESQUISADORES DO INSTITUTO BUTANTAN (1988)

1. ALMEIDA, M.E.S. de; MARTINI, M.H.; PORTO, E.; CAMARGO, A.M.M. de; RIZZO, E. de; LACAZ, C.S.
Identificação da microbiota fúngica de ambientes considerados assépticos.
Rev. Saúde públ., S. Paulo, *22* (3): 201-6, 1988.

Resumo: Objetivou-se isolar e identificar a microbiota fúngica em ambientes considerados assépticos, através de exposições com meios de cultivo adequados, em três épocas distintas do ano, antes e imediatamente após as manobras técnicas realizadas em três áreas de trabalho: ambiente aberto, ambiente fechado sem filtração de ar e ambiente fechado com filtração de ar, utilizadas em produção de imunobiológicos. Os meios ágar-Sabouraud e ágar-soja, enriquecidos com 0,2% de extrato de levedura e sem cloranfenicol, foram estudados quanto à sua eficácia no isolamento de bolores e leveduras, considerando-se o número de colônias desenvolvidas e a freqüência dos diversos fungos isolados. Isolaram-se 67 espécimens, sendo 64 fungos filamentosos (bolores) e três leveduras. Dos bolores, 54 pertenciam a 22 gêneros da divisão *Deuteromycota*, famílias *Moniliaceae e Dematiaceae*, cinco amostras filamentosas foram incluídas na ordem *Agonomycetales (Mycelia Sterilia)*, e uma amostra foi classificada na divisão *Deuteromycota*, ordem *Sphaeropsidales*, classe *Coelomycetes*. Da divisão *Zygomycota*, ordem *Mucorales*, família *Mucoraceae*, um único mucoráceo foi identificado até gênero. As três leveduras pertenciam também à divisão *Deuteromycota (Fugi Imperfecti)*, família *Cryptococcaceae*, e foram identificadas como sendo duas *Rhodotorula rubra* e uma *Torulopsis candida*. Comprovou-se que o número de colônias isoladas aumentou após a realização das manobras técnicas e que a filtração de ar através de filtros tipo HEPA, reduzindo o número de colônias isoladas nos ambientes fechados, aumenta a segurança do trabalho; comumente é recomendada para áreas de atividade técnica cujos resultados satisfatórios estão diretamente relacionados com uma baixa incidência de contaminantes.

Abstract: The isolation and identification of the fungic microbiota present in areas considered aseptic (open and/or restricted, with or without air filtration (HEPA filters) where production of immunobiologicals is carried out) were investigated. Three exposures were made with appropriate culture media, in different seasons of the year, before and immediately after the performance of the technical work. Agar-Sabouraud and agar-soy media supplemented with 0.2% yeast extract and with-out chloranphenicol had their efficacy tests in isolating the filamentous fungiae (molds) and yeasts in the light of the number of cultures grown and the frequency exhibited by the various specimens isolated. Sixty-seven specimens (64 of filamentous fungiae (molds) and three yeasts) have been identified. Of the filamentous fungiae (molds), 54 belonged to 22 genera of the division *Deuteromycota*, families *Moniliaceae* and *Dematiaceae*, five were included in the order *Agonomycetales (Mycelia Sterilia)*, and one was classified in the order *Sphaeropsidales*, class *Coelomycetes*. Of the division *Sygomycota*, order *Mucorales*, family *Mucoraceae*, only one specimen could be identified as to genus. The three yeasts, belonging also to the division *Deuteromycota (Fungi Imperfecti)*, family *Cryptococcaceae*, were identified as two *Rhodotorula rubra* and one *Torulopsis candida*. It was demonstrated that the number of colonies isolated in the areas studied increased after the performance of technical maneuvers and that air filtration by means of HEPA filters, reducing to number of colonies isolated in restricted areas, increased safety and is consequently recommended for working areas where successful or unsuccessful results depend on a low incidence of contamination.

2. BEÇAK, M.L.; SANTOS, R.C.S. dos; SOARES-SCOTT, M.D.; BATISTIC, R.F.; COSTA, H.
Chromosome structure in man and amphibia-anura, restriction enzymes.
Rev. bras. Genet., 11 (4): 939-48, 1988.

Abstract: Fixed metaphase chromosomes from human and from polyploid anurans *(Odontophrynus americanus*, 4n = 44), were treated with MspI, Hpa II, Eco RI, Bgl II, Dra I and Hae III, restriction enzymes, followed by Giemsa staining. MspI (C ↓ CGG) and Hae III (GG ↓ CC) produced G-like bands in human chromosomes indicating that the bands are caused by the removal of CG-rich specific DNA classes. However, comparable G-band patterns were also obtained in human chromosomes with Eco Ri (G ↓ AATTC),Bgl II (A ↓ GATCT) and Dra I (TTT ↓ AAA). Whose digesting sites do not reveal a particular CG-richness. In the 4n anurans, MspI-removed chromosomal DNA from the long arm of two to three homologues of group I (1q), from the secondary constriction of group 11 as well as from centromeric, telomeric and intercalary C bands. These C bands were not removed with Hpa II (C ↓ CGG), which does not cut methylated DNA. Eco RI caused bands on 2p and 3q. The results suggest that: 1. the action of these enzymes depends on the accessibility of the enzymes to chromosome regions of different DNA packaging. 2. The difficulty in obtaining interstitial bands in anurans may result from the smaller amount of CG-rich sequences probably arranged in small clusters or from the higher level of chromatin packaging in the chromomeres.

Resumo: Cromossomos metafásicos fixados de humano e de anuros poliplóides *(Odontophrynus*

americanus, 4n =44) foram tratados com enzimas de restrição MspI, Hpa II, Eco RI, Bgl II, Dra I e Hae III, seguidos pela coloração Giemsa. MspI (C ↓ CGG) e Hae III (GG ↓ CC) produziram bandas similares a G nos cromossomos humanos, indicando que as bandas são causadas pelas remoções de classes específicas de DNA ricas em CG. Contudo, padrões comparáveis de banda G foram também obtidos nos cromossomos humanos com Eco RI (G ↓ AATTC), Bgl II (A ↓ GATCT) e Dra I (TTT ↓ AAA), cujos sítios de digestão não revelam abundância particular em CG. Nos anuros 4n, MspI removeu DNA cromossômico do braço longo de dois a três homólogos do Grupo I (1q), da constricção secundária do grupo 11 bem como as bandas centroméricas, teloméricas e intercalares. Essas bandas não foram removidas pela Hpa II (C ↓ CGG), a qual não corta DNA metilado. Eco RI causou bandas 2q e 3q. Os resultados sugerem que: 1. a ação dessas enzimas depende da acessibilidade da enzima a regiões cromossômicas com compactações diferentes do DNA; 2. a obtenção difícil de bandas intersticiais em Anura pode resultar da menor quantidade de seqüências ricas em CG provavelmente arranjadas em cachos menores, ou da maior condensação cromatínica nos cromômeros.

3. BRODSKYN, C.I.; SILVA, A.M.M. da; TAKEHARA, H.A.; MOTA, I.
Characterization of antibody isotype responsible for immune clearance in mice infect with Trypanosoma cruzi.
Immunology Letters, 18: 255-58, 1988.

Abstract: Humans and mice chronically infected with *Trypanosoma cruzi* present a strong humoral immune response mediated by specific antibodies. Passive transfer of homologous immune serum to normal mice containing circulating bloodstream trypomastigotes (Bts) induces a very fast clearance of the parasites. In order to find out the role of the different immunoglobulin classes in the clearance mice containing a known number of these parasite forms in circulation were injected with total immune serum, IgG-free serum, IgG1, or IgG2 fractions and the speed of removal of the parasites from circulation was determined. The results of these experiments suggest that the immune clearance of *T. cruzi* is due to antibodies located in the IgG isotype, particularly in the IgG2 subclass.

Resumo: Homens e camundongos com infecção crônica com *T. cruzi* apresentam uma forte resposta humoral com níveis elevados de anticorpos anti-*T. cruzi*. Transferência passiva de soro imune homólogo para camundongos normais contendo tripomastigotas sangüíneos circulantes causa uma rápida remoção dos parasitas da circulação. Com a finalidade de verificar a importância das diferentes classes de imunoglobulinas neste fenômeno, camundongos com um número conhecido de tripomastigotas na circulação foram com soro imune total ou com as frações IgG1 ou IgG2 e a velocidade de remoção dos parasitas circulantes foi determinada e comparada em cada situação. Os resultados destes experimentos sugerem que a remoção imune do *T. cruzi* é devido a anticorpos localizados na classe IgG, particularmente na subclasse IgG2.

4. CASTRO, N.H.C.; WALTER, J.; SANTOS, R.C.S.; D'ANGELINO, J.L.; BENESI, F.; BIRGEL, E.H.; BEÇAK, W.
Cytogenetic study of cattle affected by persistent lymphocytosis.
J.Vet.Med. A, 35: 380-4, 1988.

Abstract: Eight BLV-seropositive female cattle presenting persistent lymphocytosis associated with BLV-infection were submitted to cytogenetic analysis. As a control group, six BLV-seronegative females, from the same herd, with no haematological or clinical changes were used. The affected group revealed a significant increase in the proportion of aneuploid cells with chromosome aberrations. An immunological assay revealed that the cells presenting the chromosomal aberrations were from the B cell population. The question of a direct or indirect action of the virus on the chromosomes in causing these changes is discussed.

Resumo: Foram analisadas citogeneticamente 8 fêmeas bovinas soropositivas quanto ao vírus da leucose bovina (BLV), que apresentavam quadro de leucocitose persistente. Constituíram o grupo controle 6 fêmeas soronegativas quanto ao BLV, da mesma fazenda, sem alterações clínicas ou hematológicas. A proporção de células aneuplóides com aberrações cromossômicas encontrada nos animais infectados pelo BLV foi significantemente maior que no grupo controle. Através de teste imunológico verificou-se que as células portadoras de aberrações cromossômicas pertenciam a população de células B. A ação direta ou indireta do vírus, como agente causador das alterações cromossômicas detectadas, é discutida.

5. CIPOLLA-NETO, J.; AFECHE, S.C.; MENNA-BARRETO, L.; MARQUES, N.; BENEDITO-SILVA, A.A.; FORTUNATO, G.; RECINE, E.G.I.G.; SCHOTT, C.
Lack of similarity between the effect of lesions of the suprachiasmatic nucleus and subparaventricular hypothalamic zone on behavioral circadian rhythms.
Brazilian J. Med. Biol.Res., 21: 653-4, 1988.

Abstract: Rats were submitted to eletrolytic lesion of either the suprachiasmatic nucleus (SCN) or the subparaventricular hypothalamic zone (SPVH) and the effects on circadian behavioral rhythms were compared. While the SCN lesion abolished the circadian rythmicity of all behavioral patterns, the SPVII lesion only abolished that of the eating and drinking behavior and reduced the amplitude of a behaviora item usually associated with REM sleep.

Resumo: Foram realizadas lesões eletrolíticas bilaterais dos núcleos supraquiasmáticos (NSQ) ou

da região hipotalâmica subparaventricular (RHSP) em ratos e comparados os efeitos destas lesões sobre os ritmos circadianos comportamentais. A lesão dos NSQ aboliu a ritmicidade circadiana de todos os padrões comportamentais, enquanto a lesão da RHSP aboliu apenas a ritmicidade circadiana dos comportamentos de comer e beber e reduziu a amplitude de um item comportamental geralmente associado ao sono paradoxal.

6 .CIPOLLA-NETO, J.; NEGRÃO, N.; AFECHE, S.C.; PALUDETTI, L.A.; BENEDITO-SILVA, A.A.; MARQUES, N.; MENNA-BARRETO, L.
Remarkable similarities between the temporal organization of neocortical electrographic sleep patterns of rats and humans.
Brazilian J.Med.Biol.Res., 21: 599-601, 1988.

Abstract: Electrocorticographic activity was automatically recorded in albino rats for 72 consecutive h and analyzed by procedures suitable to detect 24-h rhythms. Beta (alert wakefulness), theta (somnolence), delta (slow wave sleep) and sigma I (superficial synchronized sleep) activities showed a robust circadian rhythmic distribution. The acrophases (maxima of the adjusted cosine curve) occured at 23:39,07:59, 08:37 and 13:25h, respectively. EMG atonia and extreme hypotonia (less than 10% of mean EMG level) episodes showed a 24-h rhythm peaking at 14:18 h. The temporal sequence within the circadian rest period, i.e., somnolence, show wave sleep, superficial synchronized sleep and paradoxical sleep, is very similar to that known to occur during the nocturnal sleep of humans.

Resumo: A atividade eletrocorticográfica de ratos albinos foi registrada automaticamente por 72h consecutivas e, posteriormente, analisada por procedimentos adequados para detectar a presença de ritmos de 24 h. As atividades beta (vigilia com alerta), teta (sonolência), delta (sono de ondas lentas) e sigma I (sono sincronizado superficial) apresentaram uma distribuição circadiana evidente. As acrofases (ponto máximo da curva cosseno ajustada) ocorreram às 23:39; 07:59; 08.39 e 13:25h, respectivamente. Os episódios de atonia e hipotonia extrema (menos do que 10% do nível médio do EMG) apresentaram um ritmo de 24h com um pico às 14:18h. A seqüência temporal das diversas fases do sono dentro do período circadiano de repouso, isto é, sonolência, sono de ondas lentas, sono sincronizado superficial e sono paradoxal, é muito semelhante à que ocorre durante o sono noturno em humanos.

7. CONSALES, C.A; VALENTINI, E.J.G.; ALBAS, A., MENDONÇA, R.M.Z.; FUCHES, R.M.M.; SOARES, M.A.; PEREIRA, C.A.
The preparation of cultured rabies virus and the production of antiserum for human use.
J.biol.Stand., 16: 27-32, 1988.

Abstract: In this paper we describe a methodology for the preparation of the Pasteur strain of fixed rabies virus in BHK-21 clone 13 cells and also its use for the production of antisera in horses. The methodology showed here is simple, rapid, facilitates the attainment of high protective titers, and the antisera produced are of high quality.

Resumo: Descrevemos uma metodologia para o preparo da cepa Pasteur de virus rábico em células BHK-21 e o seu uso na preparação de antisoro em cavalos. A metodologia é simples, rápida, permite a obtenção de altos títulos de anticorpos e um soro de alta qualidade.

8. *CORTADAS, J. & RUIZ, I.R.G.*
The organization of ribosomal genes in diploid and tetraploid species of the genus Odontophrynus (Amphibia, Anura)
Chromosoma, 96: 437-42, 1988.

Abstract: The organization of the repeat unit of the ribosomal genes was determined in populations of Odontophrynus americanas 2n, 4n, O. occidentalis 2n and *O. barrioi 2n* (Amphibia, Anura) from South America. HindIII, EcoRI and BamHI restriction patterns of rDNA from single individuals were probed with two rDNA clones from *Xenopus laevis*. Variations in length and sequence were found among different species, populations and specimens. A general pattern could be established from the most frequent type of repeat observed in each case, so that diploid populations could be distinguished from tetraploid ones. The molecular evolution of variant repats is discussed. It is suggested that the reduced size of the non-transcribed spacers in the tetraploids from São Paulo could play a role in the control of rDNA transcription. Distinct restriction maps were tentatively correlated to the varying localization of active rDNA clusters on metaphase chromosomes of corresponding populations.

Resumo: A organização da unidade repetitiva dos genes ribossômicos foi determinada em populações de *O. americanus* 2n e 4n, *O. occidentalis* 2n, e *O. barrioi* 2n (Amphibia, Anura) da América do Sul. Os padrões de restrição obtidos com as enzimas Hind III, Eco Ri, e Bam HI a partir de DNA total de cada indivíduo foram sondados com dois clones de rDNA de *Xenopus laevis. Encontramos variações no comprimento e seqüência, das unidades repetidas entre diferentes espécies, populações e espécimens. Um modelo geral pôde ser estabelecido baseado no tipo de maior freqüência de repetição observada em cada caso, de modo que as populações diplóides podiam ser distinguidas das tetraplóides. Discute-se a evolução molecular da variante repetitiva. Sugere-se que a redução de tamanho do espaçador (NTS) nos tetraplóides de São Paulo poderia desempenhar um papel no controle da transcrição do rDNA. Correlacionamos os diferentes mapas de restrição com as lo-*

calizações variadas de agrupamentos de rDNA ativos em cromossomos metafásicos de populações correspondentes.

9. *DENARO-MACHADO, L.; MACHADO, J.C.; LONGO, M.A; OGATA, T.R.P.; DAVID, M.L.V.M.G.; KFOURI, S.A.; CARNEIRO, S.M.*
Heterotransplante de células KB em ratos nude atímicos. II. Estudos morfológicos.' Acta Oncol. Bras., 8: 21-5, 1988.

Resumo: Estudos morfológicos de microscopia óptica e eletrônica foram realizados em tumores crescidos em ratos nude atímicos, pós-transplantes de linhagem celular tumoral humana de carcinoma epidermóide de boca (KB), desde a 1ª inoculação realizada, até a 8ª passagem seriada do tumor nesses animais. Os autores descrevem o aumento do volume celular e nuclear, bem como a elevação do número de mitoses e uma maior velocidade de crescimento da massa tumoral, à medida que as passagens seriadas do tumor se sucedem. Também foi observado que, com o progredir dessas passagens, a reação inflamatória por parte do hospedeiro aumenta.

Abstract: Morphological studies of optical and electronic microscopy were done in tumors grown in athymic nude rats transplanted with human tumor cell line derived from epidermoid mouth carcinoma (KB), since the first inoculation till the 8th serial passage of the tumor in these animals. The authors describe the increase of the cellular and nuclear volume, as well as the increase of the number of mitoses and a greater speed of growth of the tumoral mass throughout the serial passages. Also, with the progress of the passages, it has an increase of the inflammatory reaction of the host.

10. DIAS, R.M.D.S.; MANGINI, A.C.S.; TORRES, D.M.A.G.V.; CORREA, M.O.A.; LUPETTI, N.; CORREA, F.M.A.; CHIEFFI, P.P.
Cryptosporidiosis among patients with acquired immunodeficiency syndrome (AIDS) in the country of São Paulo, Brazil.
Rev. Inst. Med. trop. São Paulo, 30 (4): 310-2, 1988.

Abstract: Stool samples of 157 patients with AIDS, living in the country of São Paulo, were submitted to several techniques in the search for *Cryptosporidium* sp. Among the various techniques tested for slide preparation (direct smear, spontaneous sedimentation method, and formol-ether concentration), the latter, formol-ether concentration, offered the best results, clearly outdoing all the others. Nineteen samples out of 157 prepared by this technique, after dyeing by the Kinyoun method or by carbol fuchsin dimethyl sulfoxide, were found to be positive for *Cryptosporidium* sp.

Resumo: Amostras de fezes de 157 pacientes acometidos pela síndrome de imunodeficiência adquirida, residentes no município de São Paulo, foram submetidas a diversas técnicas para pesquisa de *Cryptosporidium* sp. Entre as diversas técnicas testadas para o preparo das lâminas (esfregaço direto, método de sedimentação espontânea e concentração pelo formol-éter) a concentração pelo formol-éter foi a que forneceu melhores resultados, superando nitidamente as demais. Nas 157 amostras preparadas por esta técnica, após coloração pelo método de Kinyoun ou pela fucsina-carbólica dimetilsufóxido, encontraram-se 19 (12,1%) positivas para oocistos de *Cryptosporidium* sp.

11. DORCE, V.A.C. & PALERMO-NETO, J.
Effects of ovariectomy and of estrogen administration on some dopamine-related behaviors in the rat.
Brazilian J. Med. Biol. Res., 21: 313-320, 1988.

Abstract: 1. The effects of ovariectomy and of estrogen administration were determined on some dopamine-related behaviors.
2. Haloperidol withdrawal induced a progressive increase in locomotion and rearing frequencies of female rats observed in an open-field. This increase started earlier and was greater in ovariectomized than in sham-operated animals.
3. Repeated administration of increasing doses of 17- -estradiol (from 0.05 to 0.3mg/kg once daily, for 28 days) significantly decreased apomorphine-induced stereotyped behavior in ovariectomized rats, 30 min after the last hormone injection.
4. Withdrawal from long-term 17- -estradiol administration increased the sensitivity of the animals to apomorphine.
5. These results suggest not only an antidopaminergic effect for estrogen but also a possible summation of estrogen and haloperidol withdrawal effects.

Resumo: 1. Os efeitos da castração e da administração do estrógeno foram determinados em comportamentos relacionados aos sistemas dopaminérgicos.
2. A retirada do haloperidol induziu um aumento progressivo na freqüência de locomoção e de levantar de ratas observadas no campo-aberto. Esse aumento começou logo após a retirada da droga e foi mais intenso em ratas ovariectomizadas que em ratas falso-operadas.
3. A administração de drogas crescentes de 17- -estradiol (de 0,05 a 0,3 mg/kg uma vez ao dia, por 28 dias) diminui significativamente o comportamento esteriotipado induzido pela apomorfina em ratas ovariectomizadas, 30 minutos após a última injeção do hormônio.
4. A retirada da administração prolongada do 17- -estradiol aumenta a sensibilidade dos animais à apomorfina.
5. Esses resultados sugerem não só um efeito dopaminérgico para o estrógeno mas também uma possível somação dos efeitos da retirada do estrógeno e do haloperidol.

12. KIKUCHI, O.K.; OKAZAKI, K.; KAWANO, T.; RIBEIRO, A.A.G.F.C.
Estudos ultra-estruturais de embrião de *Biomphalaria glabrata* Say, 1818.
Publicação IPEN, (203): 1-10, 1988.

Resumo: Os estudos ultra-estruturais de embriões de *Biomphalaria glabrata* (Mollusca: Gastropoda), um importante caramujo vetor da esquistossomose, não têm sido explorados. No presente trabalho foi avaliada a técnica mais adequada para o processamento dos embriões para a microscopia eletrônica. A técnica que forneceu resultados bastante promissores foi a fixação dupla em glutaraldeído 1% mais tetróxido de ósmio 1% em tampão cacodilato 0,05 M (pH 7,4) a 4°C, pré-contraste em acetato de uranila 1% durante uma noite e a embebição tanto em resina EPON como Polylite sob vácuo. Foram utilizados embriões no estádio de trocófora jovem que se caracteriza pela intensa organogênese. Alguns aspectos da ultra-estrutura de células embrionárias de *B. glabrata* são apresentados.

Abstract: Ultrastructural studies of *Biomphalaria glabrata* embryos (Mollusca: Gastropoda), an important snail vector of schistosomiasis, has not been explored. In the present work it was evaluted a suitable electron microscopical technique for embryos processing. Promising results was obtained with double fixation in 1% glutaraldehyde plus 1% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.4), preliminary staining overnight in 1% uranyl acetate and embedding in EPON or Polylite under vacuum. It was used embryos at young trochophore stage wich is characterized by active organogenesis. Some ultrastructural aspects of *B. glabrata* embryos cells are presented.

13. LEBRUN, I.; CAMARGO, A.C.M.; CORREA, F.M.A.
Pharmacological effects and metabolism of neurotensin and bradykinin in the isolated rat uterus.
Europ. J. Pharmacol., 148: 231-7, 1988.

Abstract: Neurotensin (NT) and bradykinin (BK) were found to cause contractions of isolated rat uterus preparations. In 97% of the experiments, acute tachyphylaxis followed soon after the initial administration of NT. Interrelation between the oxytocic effects of NT and BK was not observed. Among the NT fragments studied, only NT-(9-13) had an oxytocic effect (1.0%). All NT fragments tested induced tachyphylaxis to NT regardless of their efficacy. Using HPLC analysis, NT but nct BK was found to be degraded by the intact rat uterus. A major involvement of a carboxydipeptidase cleaving at Tyr^{11}-Ile^{12} is suggested. Carboxyl-blocked neurotensinamide (NT-NH_2) was found to be resistent to proteolysis and not develop tachyphylaxis. No cross-tachyphylaxis was observed between NT and NT-NH_2. The oxytocic effects of NT-NH_2 were markedly potentiated by pre-incubation with NT or NT-(1-11). The results suggest the existence of different receptors for NT and BK in the uterus, as well as the existence of different receptors or receptor states that interact with NT or NT-NH_2 in the rat uterus.

Resumo: Tanto a Neurotensina (NT) quanto a Bradicinina (BK) induzem contração em preparações de útero isolado de rata. Verificou-se que em 97% dos experimentos houve o desenvolvimento de uma taquifilaxia aguda após a administração de NT. Não se verificou uma interrelação entre os efeitos acitócicos da NT e BK. Estudando-se fragmentos da NT, somente NT_{9-13} possui um efeito acitócico (1%). Todos outros fragmentos testados também induziram taquifilaxia, utilizando-se análise em HPLC verificou-se que a NT mas não a BK é degradada pela preparação intacta do útero isolado de rata. Sugere-se um maior envolvimento de uma carboxidipeptidase clivando o peptídeo em Tir^{11} — $11e^{12}$. A neurotensinamida, um análogo carboxil-bloqueado, foi resistente à proteólise e não desenvolveu taquifilaxia. Não se observou taquifilaxia-cruzada entre NT e NT-NH_2. Os efeitos acitócicos da NT-NH_2 foram marcadamente potenciados pela pré-incubação com NT ou NT (1-11). Os resultados sugerem a existência de diferentes receptores para a BK e NT no útero, bem como a existência de diferentes conformações de receptores que interagem com NT ou NT-NH_2.

14. LEBRUN, I.; LEBRUN, F.L.A.S.; AIZENSTEIN, M.L.
Evidence for a role of B-Endorphin in activity of nigrostriatal neurons in the rat.
Brazilian J. Med. Biol. Res., 21: 645-7, 1988.

Abstract: The effects of *icv* administration of β -endorphin on secretory activity of dopaminergic neurons is described. Homovanilic and dihydroxyphenyl acetic acid levels in cerebrospinal fluid and extracts of brain tissue were determined after administration of β -endorphin to animals pretreated or not with naloxone. The results suggest that β -endorphin interferes with formation of dopaminergic metabolites by acting on opioid receptors.

Resumo: Foram estudados os efeitos da administração i.c.v. de β-endorfina na atividade secretória dos neurônios dopnérgicos.
Os níveis dos ácidos homovanílico e dihidroxifenilacético foram determinados após a administração de β-Endorfina no fluido cérebro-espinhal e extratos de tecido cerebral em animais pré-tratados ou não com naloxone. Os resultados sugerem que a β-Endorfina interfere com a formação dos metabólitos da dopamina através da ação em receptores apióides.

15. LUCAS, S.
Spiders in Brazil.
Toxicon, 26 (9): 759-72, 1988.

Abstract: Descriptions of the principal venomous spiders in Brazil, of the genera *Phoneutria, Loxosceles, Latrodectus* and *Scaptocosa,* are given, together with a list of species and their geographical distribution. Detailed information on their habitat, behaviour and venom quantity, symptomatology of human accidents and epidemiology is given and recommendations are made for the prevention of accidents. General observations are made on the most common mygalomorph spiders.

Resumo: São fornecidas descrições das principais aranhas peçonhentas brasileiras pertencentes ao gênero *Phoneutria, Loxosceles, Latrodectus e Scaptocosa;* uma lista das especies e sua distribuição geográfica. Informamos sobre o habitat, comportamento, quantidade de veneno, sintomatologia dos acidentes humanos, dados epidemiológicos. São dadas recomendações sobre prevenção dos acidentes e informações gerais sobre as aranhas MYGALOMORPHAE mais comuns.

16. MANCINI, D.A.P.; GERALDES, E.A.; PINTO, J.R.; SOARES, M.A.
Ocorrência de influenza eqüina na Fazenda São Joaquim, São Roque-SP, do Instituto Butantan.
Rev.Fac.Med.Vet.Zootec.Univ.S.Paulo, 25 (1): 93-100, 1988.
*Resumo:*Foi verificada a incidência de influenza eqüina através da positividade de respostas de anticorpos contra o vírus influenza, cepa A/Eq^2, em soros de cavalos pertencentes ao Setor de Imunizações da Fazenda São Joaquim, do Instituto Butantan. Foi observada pequena diferença nas respostas de anticorpos, tanto no rebanho de produção, como de controle de imunoterápicos; ambos tiveram decréscimo de títulos de anticorpos no período de 80 dias após o surto. A queda de nível de anticorpos protetores verificada na análise sorológica dos cavalos indica a baixa proteção que possuem contra a infecção ou reinfecção.

Abstract: The prevalence of equine influenza was verified by means of the positivity of the antibody responses to the influenza virus strain A/Eq_2, on sera taken from horses belonging to São Joaquim Farm, Immunizations Section, of the Instituto Butantan, placed in the State of São Paulo. Little differences were observed in antibody responses between horses designated for production and control of immunotherapeutic agents there was, however a decrease of antibody titles in the period of 80 days after the outbreak. The drop in the level of the antibodies observed by serologic analysis of those horses, indicates the low protection conferred by the antibodies against infection or reinfection.

17. MANDELBAUM, F.R. & ASSAKURA, M.T.
Antigenic relationship of hemorrhagic factors and proteases isolated from the venoms of three species of *Bothrops* snakes.
Toxicon, 26 (4): 379-85, 1988.

Abstract: By comparative studies of the immunological properties of the metalloproteins (hemorrhagic factors and proteases) isolated from the venoms of *Bothrops jararaca, Bothrops neuwiedi* and *Bothrops moojeni*, it was found that the hemorrhagic factors contain common antigenic determinants and the proteases were immunologically distinct entities. The rabbit antisera raised for the hemorrhagic factors not only neutralized the hemorrhagic activities of the respective factors but also activities of the other hemorrhagic factors. Although the homology among these proteins are not yet known, these studies have shown that the hemorrhagic factors must have a similar partial structure which includes the catalytic hemorrhagic active site.

Resumo: Estudos comparativos das propriedades imunológicas de metaloproteínas (fatores hemorrágicos e proteases) isolados dos venenos de *Bothrops jararaca, Bothrops neuwiedi* e *Bothrops moojeni*, mostraram que os fatores hemorrágicos contêm determinantes comuns enquanto que as proteases são imunologicamente distintas. Os antissoros específicos produzidos em coelhos contra os fatores hemorrágicos, foram capazes de neutralizar tanto a atividade hemorrágica dos respectivos fatores como as atividades dos outros fatores hemorrágicos. Embora a estrutura molecular destas proteínas ainda não seja conhecida, estes estudos mostraram que os fatores hemorrágicos devem apresentar homologia parcial correspondente ao sítio catalítico da molécula.

18. MANDELBAUM, F.R.; REICHL, A.P.; ASSAKURA, M.T.
Hemorrhagic factors from the venoms of two species of *Bothrops* snake.
SIMPÓSIO ANUAL DA ACIESP SOBRE TOXINAS PROTEÍCAS, 12. 1988, v.1, p. 9-24, 1988.

Abstract: From two venoms of *Bothrops* species were isolated five hemorrhagic factors, NHF_a and NHF_b from the venom of *B. neuwiedi* and HF_1, HF_2 and HF_3 from the venom of *B. jararaca*. Like the other hemorrhagic principles isolated from snake venoms, they are heat labile metalloproteins, unstable at acidic medium and inhibited by metal chelator compounds such as EDTA, EGTA and 1.10-phenanthroline. They have no action on arginine synthetic substrates and they are not affected by inhibitors of serine proteases such as DFP, TLCK and TPCK. They are acidic proteins whose pIs range from 3.9 to 4.3 and the molecular weights range from 46,000 to 62,000. The minimum hemorrhagic dose (MHD), the amount of protein which produces a hemorrhagic spot of 1 square cm on rabbit skin was used to compare activities of these factors. NHF_b, NHF_a, HF_3, HF_2 and HF_1 required 0.2, 4.8, 15,20 and 100ng of protein, respectively. There are also differences concerning the action on casein. The proteolytic action of HF_2 and NHF_a were higher than the others. The hemorrhagic factors hydrolyze similarly the B-chain of oxidized insulin. The hydrolysis occurs preferen-

cially, in bonds where leucine and phenylalanine contribute with the amino group. Comparative studies of these isolated hemorrhagic factors with horse monovalent antivenoms revealed that they were not identical but immunologically related proteins. This relation ship was confirmed with rabbit specific antisera raised to these hemorrhagic factors. Also the specific antisera not only neutralized the hemorrhagic activities of the homologous proteins, but also the hemorrhagic activities of the heterologous isolated factors. These specific sera were also able to recognize hemorrhagic proteins and neutralize partially the hemorrhagic activity in several venoms of the Crotalinae subfamily.

Resumo: Do veneno de B. jararaca foram isolados os fatores hemorrágicos HF_1. HF_2 e HF_3 e do veneno de B. neuwiedi NHF_a e NHF_b. Semelhantemente a outros fatores hemorrágicos isolados de venenos de serpentes, estes são metaloproteases instáveis a pHs ácidos e inibidas por compostos quelantes tais como EDTA, EGTA e o-fenantrolina. Essas proteases não agem sobre substratos sintéticos da arginina e não são afetadas por inibidores de serina proteases tais como DFP, TLCK e TPCK. São proteínas ácidas cujos pls variam de 3,9 a 4,3 e seus pesos moleculares são da ordem de 46.000 a 62.000. A D.M.H. (dose mínima hemorrágica), que corresponde à quantidade de proteína necessária para produzir uma área hemorrágica de $1cm^2$ na derme de coelho, foi de 0,2; 4,8; 15; 20 e 100 ng para NHF_b, NHF_a, HF_3, HF_2 e HF_1, respectivamente. Igualmente na atividade sobre a caseína foram detectadas algumas diferenças. A ação proteolítica de HF_2 e NHF_a foi maior do que os outros fatores. Esses fatores hemorrágicos hidrolisam semelhantemente a cadeia B de insulina oxidada. A hidrólise ocorre preferencialmente nas ligações onde leucina e fenilalanina participam com o grupo amino. Estudos comparativos desses fatores hemorrágicos usando antivenenos produzidos em cavalos mostraram que não são proteínas idênticas mas imunologicamente relacionadas. Estes dados foram confirmados por soros anti-hemorrágicos específicos produzidos em coelhos. Esses antissoros neutralizaram a atividade hemorrágica do fator homólogo, bem como dos outros fatores hemorrágicos isolados. Estes soros específicos ainda foram capazes de reconhecer proteínas hemorrágicas e também neutralizar parcialmente a atividade hemorrágica de vários venenos da subfamília Crotalinae.

19. MARQUES, N.; AFECHE, S.C.; BENEDITO-SILVA, A.A.; CIPOLLA-NETO, S.; FORTUNATO, G.; PALUDETTI, L.A.; RECINE, E.G.I.G.;SCHOTT, C.; MENNA-BARRETO, L.S.
Chronobiology courses at the University of São Paulo/Brasil; a four-year experience.
Chronobiologia, 15: 235-42, 1988.

Abstract: The Grupo Multidisciplinar de Desenvolvimento e Ritmos Biológicos — GMDRB (Multidisciplinary Group of Development and Biological Rhythms) of the University of São Paulo (USP) — is the first group in Brazil to have Chronobiology as its central topic of research.
Besides research activities the group are involved with the spread of basic information on Chronobiology through graduate and post — graduate courses.
This article describes the didatic experience on graduate and post — graduate courses on Chronobiology in São Paulo, Brazil.

Resumo: O Grupo Multidisciplinar de Desenvolvimento e Ritmos Biológicos (GMDRB) do Instituto de Ciências Biomédicas da USP é o primeiro Grupo brasileiro que tem como tema central de pesquisa a Cronobiologia. *Além dos trabalhos de pesquisa o grupo desenvolve também atividades de divulgação de informações básicas sobre cronobiologia através da realização de cursos a nível* de graduação e pós-graduação.

Este artigo descreve a experiência didática de um curso de Cronobiologia, para alunos já graduados e pós-graduandos, realizado anualmente em São Paulo, Brasil.

20. MOURA, J.W.; STOCCO DOS SANTOS, R.C; DAGLI, M.L.Z.; D'ANGELINO, J.L.; BIRGEL, E.H.; BEÇAK, W.
Chromosome aberrations in cattle raised on bracken fern pasture
Experientia, 44: 785-8, 1988.

Abstract: Thirteen cows maintained on natural bracken fern *(Pteridium aquilinum)* were analyzed cytogenetically. The frequency of structural chromosome aberrations detected in peripheral blood cells was significantly higher when compared to that detected in animals raised on pasture containing no bracken fern. We discuss the clastogenic action of fern and its synergistic action with infection by type 2 and 4 papilloma virus in the same animals.

Resumo: Analisamos citogeneticamente 13 bovinos mantidos em pastagem natural de samambaia *Pteridium aquilinum*. A freqüência de aberrações cromossômicas estruturais encontrada, a nível de sangue periférico nesses animais, é altamente significante, quando comparada a de animais de áreas sem samambaia. Discute-se a ação clastogênica da samambaia e seu efeito sinergicamente à infecção, desses animais, pelo vírus do papiloma bovino tipo 2 e 4.

21. SALOMÃO, M. da G. & SAWAYA, P.
Morphology and regeneration of the gonopodium in poecilid fish.
Zool. Anz., 220 (3/4) S.135-143, 1988.

Abstract: In the present study the development of the gonopodium was accompanied and its histology

in adult of *Poecilia reticulata* and *Phalloceros caudimaculatus* was described. Regeneration of the gonopodium in fresh water and *sea* water was examined. The gonopodium of *P. reticulata* terminates in a globular expansion while in *P. caudimaculatus* the gonopodial extremity is bifurcated. Variation with age in terminal portion of the gonopodium is marked in *P. caudimaculatus*. In both species the organ is composed of hyaline conjuctive tissue, the regeneration after sectioning is not complete and the gonopodium is not functional in the transmission of sperm.

Resumo: No presente estudo o desenvolvimento do gonopódio de *Poecilia reticulata* e *Phalloceros caudimaculatus* foi acompanhado, a histologia do órgão em animais adultos foi descrita. A regeneração do gonopódio em água doce e água do mar foi examinada. O gonopódio de *P. reticulata* tem sua parte terminal numa expansão globular, enquanto em *P. caudimaculatus* essa extremidade é bifurcada. A variação dessa terminalização em função da idade é marcante em *P. caudimaculatus*. Em ambas as espécies o órgão é composto de tecido conjuntivo hialino, a regeneração após secção não é completa e o gonopódio não é funcional na transmissão do esperma.

22. SALVADORI, D.M.F.; RIBEIRO, L.R.; PEREIRA, C.A.B.; BEÇAK, W.
Cytogenetic effects of malathion insecticide on somatic and germ cells of mice.
Mutation Research, 204: 283-7, 1988.

Abstract: Male mice dermally exposed to single or multiple treatment (5 days/2 weeks) showed that the ability of malathion to induce chromosome aberrations in somatic (bone marrow) and germ cells (primary spermatocytes) was related to the type of treatment and dose used.
Statiscally significant increases of chromosome aberrations in bone marrow cells occurred after single treatment (500 and 2000 mg/kg body wt) when chromatid gaps were included and after multiple treatment (250 and 500 mg/kg) when they were excluded. No dose-response relationships were observed for either treatment. In germ cells, malathion induced a significant increase of univalents in both types of treatment but structural chromosome aberrations were induced only by multiple treatment.
Malathion induced a significat decrease of the mitotic indices in the bone marrow.

Resumo: Camundongos machos expostos na derme a tratamentos simples ou múltiplos (5 dias/2 semanas) mostraram que a habilidade do malation em induzir aberrações cromossômicas em células somáticas (medula óssea) e germinativas (espermatócitos primários) está relacionada ao tipo de tratamento e dose usada.
Aumentos estatísticos significantes de aberrações cromossômicas em células da medula óssea ocorreram após tratamento único (500 e 2000 mg/kg peso) quando as falhas cromatídicas foram incluídas e após tratamento múltiplo (250 e 500 mg/kg) quando elas eram excluídas. Não foram encontradas relações dose-resposta para qualquer tratamento. Em células germinativas o malation induziu um aumento significante de univalentes em ambos tipos de tratamento mas aberrações cromossômicas estruturais foram induzidas somente por tratamento múltiplo.
O malation induziu um decréscimo significante dos índices mitóticos na medula óssea.

23. SERTIÉ, J.A.A.; BASILE, A.C.; PANIZZA, S.; MATIDA, A.K.; ZELNIK, R.
Pharmalogical assay of *Cordia verbenacea;* Part 1. Anti-inflammatory activity and toxicity of the crude extract of the leaves.
Planta médica, (1): 7-10, 1988.

Abstract: Anti-inflammatory effects and toxicity of lyophilized extracts of the leaves of *Cordia verbenacea* DC (Boraginaceae) were investigated through various experimental models in rats. The crude drug significantly inhibited the carrageenin-induced edema proportionally to doses ranging from 0.59 to 2.98 mg/kg.Repeated administration of the extract at doses of 1.24 mg/kg for 6-d period similarly reduced the granuloma formation. This response appears indentical to the one from calcium phenylbutazone. The same dose of the crude extract reduced the vascular permeability response to histamine. Toxicological experiments indicated a very low toxicity, confirmed by sub-acute tests, the LD_{50} value (LD_{50} = 131.6 mg/kg) being 100 times that of the ED_{50} (ED_{50} = 1.24 mg/Kg).

Resumo: Os efeitos anti-inflamatório e toxicidade dos extratos brutos das folhas de *Cordia verbenacea* DC (Boraginacea) foram investigados por vários modelos experimentais em ratos. O extrato bruto inibiu, significativamente, o edema induzido por carrageenina. A administração repetida do extrato reduziu a formação de granuloma e resposta da permeabilidade vascular à histamina. Os ensaios toxicológicos indicaram uma baixa toxicidade.

24. SILVA, A.M.M. da; COSTA, H.H.; TAKEHARA, H.A.;MOTA, I.
Trypanosoma cruzi: advantagens of isolating bloodstream trypomastigotes by the carboxy methyl cellulose method.
Trans. roy Soc. trop. Med. Hyg., 82: 715-8, 1988.

Abstract: Bloodstream trypomastigotes were isolated from blood of A/Sn mice 7 d after infection with 10^5 *Trypanosoma cruzi* Y strain. Red blood cells were removed by centrifugation and hypotonic shock and platelets and leucocytes by passage through a carboxy methyl cellulose column. Binding of trypomastigotes to the resin was prevented by including 10% normal mouse serum in the eluting buffer. Im such conditions, more than 90% of the parasites applied to the columns were

recovered, free of white blood cells and platelets. A comparative study of the pre and post-separation trypomastigotes showed that both had the same infecting capacity, ability to evade destruction by the complement system, and antigenic profile.

Resumo: Tripomastigotas sangüíneos foram isolados do sangue de camundongos A/Sn infectados com *T. cruzi* 7 dias antes. As hemácias foram removidas por centrifugação e choque hipotônico e as plaquetas e leucôcitos por passagem através de uma coluna de carboximethil celulose. A adesão dos tripomastigotas à resina foi impedida pela inclusão de 10% de soro normal homólogo no tampão de eluição. Nestas condições mais de 90% dos parasitas colocados na coluna foram recuperados, isentos de hemácias e plaquetas. Um estudo comparativo dos tripomastigotas antes e depois da separação mostrou que ambos possuíam a mesma capacidade infectante, a mesma capacidade de resistência à lise mediada pelo C e o mesmo perfil antigênico.

25. SILVA, A.M.M. da; BRODSKYN, C.I.; TAKEHARA, H.A.; MOTA, I.
Comparison between the antigenic composition of bloodstream and cell culture-derived trypomastigotes of *Trypanosoma cruzi*.
Brazilian J.Med.Biol.Res., 21: 991-3, 1988.

Abstract: Antigens of bloodstream and cell culture-derived trypomastigotes of T. cruzi were compared by western blotting using sera of chronic chagasic patients as a source of antibodies. The immunoblots demonstrated that the two forms display extensive homology except for the 85-and 52 kDa bands. These antigens were more strongly stained in culture-derived trypomastigotes. Although the reported differences are not related to major antigens, these results might offer an explanation for previous results showing that culture derived trypomastigotes are more antigenic and infective *in vitro* than blodstream trypomastigotes.

*Resumo:*Foi feito um estudo comparativo dos antígenos obtidos de tripomastigotas sangüíneos ou de tripomastigotas de cultura por meio de ''immunobloting'' usando-se soros de pacientes chagásicos como fonte de anticorpos. Os ''immunoblots'' mostraram que as duas formas apresentam homologia intensa exceto nas bandas de 85 e 52kDa. Estes antígenos coravam-se mais fortemente nos tripomastigotas obtidos de cultura. Embora estas diferenças não sejam nos antígenos principais, estes resultados talvez ofereçam uma explicação para estudos anteriores mostrando que os tripomastigotas derivados de cultura são mais antigênicos e infectantes *in vitro* do que os tripomastigotas sangüíneos.

26. SOARES, M.F.M.; OLIVEIRA, E.B.; MOTA, I.; MACEDO, M.S.
Suppresion of IgE antibody production by *Ascaris suum* extract: characterization of suppressive component(s).
Brazilian J.Med.Biol.Res., 21: 527-9, 1988

Abstract: Partial characterization of the suppressive component(s) of *A. suum* extract that is (are) responsible for damping production of IgE antibody to ovalbumin was performed by physical and chemical methods. Digestion of the whole extract with trypsin and chymotrypsin completely abolished the suppressive activity. Oxidation with sodium metaperiodate or heating at 56°C, however, had no effect. These results indicate that the integrity of heat-stable protein(s) present in the crude extract is essential for its suppressive effect. In addition, the carbohydrate moiety does not seem to play an important role in this effect.

Resumo: Os componentes supressores do extrato de *A. suum* que agem sobre a produção de anticorpos IgE foram caracterizados por técnicas físicas e químicas. A digestão do extrato total com tripsina e quimotripsina aboliu completamente a atividade supressora. Oxidação por metaperiodato ou aquecimento a 56°C não tiveram efeito. Estes resultados indicaram que a integridade das proteinas presentes no extrato bruto é essencial para o efeito supressor e que os componentes carboidratos não parecem ter uma função importante no efeito supressor.

27. SOARES-SCOTT, MD.; TRAJTENGERTZ, I.; SOMA, M.; BEÇAK, M.L.
C. and AgAs bands of the octaploid untanha frog *Ceratophrys dorsata* (C. aurita) (8n=104, Amphibia, Anura).
Rev. bras.Genet., 11 (3): 625-31, 1988.

Abstract: Ceratophrys aurita (C. dorsata), a rare octaploid species of Brazilian anuran (8n = 104), has 9 NORs distributed among distinct chromosome groups (6 and 11) with centromeric and telomeric C bands, as analyzed in one male.
This species also presents interpopulational variability of DNA content. The NOR banding pattern shows the occurrence of interspecific polymorphism as compared to *C. ornata*. The number and distribution of NORs, as well as the variability in DNA content are caused by postploidy rearrangements.

Resumo: Ceratophrys aurita (C. dorsata), uma espécie octaplóide de anuros brasileiros, tem 9 NORs distribuídos entre dois grupos distintos de cromossomos (6 e 11) com bandas C centroméricas e teloméricas. Esta espécie apresenta variabilidade interpopulacional do conteúdo total de DNA. Seu padrão de bandamento NOR indica haver polimorfismo interespecífico, quando comparado

com *C. ornata*. O número de distribuição de NORs, bem como a variabilidade do conteúdo total de DNA foram explicados como causados por rearranjos postpoliploidia.

28. TAKEHARA, H.A.; CARDOSO, D.F.; SILVA, A.M.M. da; MOTA, I.
Lytic antibodies elicited by *Trypanosoma cruzi* infection recognize epitopes present on both bloodstream trypomastigote and epimastigote forms of parasite.
Rev.Inst.Med.trop.São Paulo,30 (5): 351-6, 1988

Abstract: Sera of Chagas'disease patients containing anti-*T. cruzi* lytic antibodies were submitted to affinity chromatography using Sepharose 4B conjugated with antigen extracted from epimastigote or trypomastigote forms of the parasite. Epimastigotes were obtained from culture at the exponential growth phase and the trypomastigotes from blood of infected and immunosuppressed mice. Antigen of both parasite forms was obtained by sonication of the parasites followed by centrifugation. Both antigens were then conjugated to activated Sepharose 4B. Affinity chromatography was performed by passing sera from chagasic patients through an immunoadsorbent column containing either epimastigotes or trypomastigotes antigens. Antibodies bound to the column were eluted with cold 0,2 M glycine buffer pH 2,8. The eluted antibodies were analysed regarding their isotype and lytic activity. The results showed that anti-*T. cruzi* lytic antibodies present in sera from chagasic patients are mainly located in the IgG isotype and recognize epitopes present in both trypomastigote and epimastigote forms.

Resumo: Soro de pacientes com doença de Chagas na fase crônica foram submetidos à cromatografia de afinidade com Sepharose 4B conjugada com um extrato antigênico obtido de formas epimastigotas ou tripomastigotas de *T.cruzi*: os epimastigotas foram obtidos de cultura na fase exponencial de crescimento e os tripomastigotas de sangue de camundongos infectados e imunossuprimidos. Os antígenos de ambas formas parasitárias foram obtidos por tratamento dos parasitas por ultra-som, seguido de centrifugação.
A cromatografia de afinidade foi feita passando-se os soros chagásicos através de uma coluna de imunoadsorvente contendo antígenos de epimastigotas ou tripomastigotas. Os anticorpos foram eluídos da coluna com tampão glicina 0,2 M pH 2,8 a 4°C. Os anticorpos eluídos foram analisados quanto ao seu isótipo e atividade lítica. Os resultados mostraram que os anticorpos anti-*T. cruzi* com atividade lítica presentes em soros chagásicos estão localizados no isótipo IgG e reconhecem epitopos presentes tanto nos tripomastigotas quanto nos epimastigotas.

29. TOFFOLETTO, O.; CAMARGO, A.C.M.; OLIVEIRA, E.B.; METTERS, K.M.; ROSSIER, J.
Liberation of enkephalins from enkephalin-containing peptides by brain endo-oligopeptidase A.
Biochimie, 70: 47-56, 1988.

Abstract: Endo-oligopeptidase A, highly purified from the cytosol fraction of bovine brain by immunoaffinity chromatography, has been characterised as a thiol endopeptidase. This enzyme, known to hydrolyse the Phe^5 — Ser^6 — bond of bradykinin and the Arg^8-Arg^9 bond of neurotensin has been shown to produce, by a single cleavage. [Leu]enkephalin or [Met]enkephalin from small enkephalin-containing peptides. Enkephalim formation could be inhibited in a concentration dependent manner by the alternative substrate bradykinin. The optimal substrate size was found to be 8-13 amino acids, with enkephalin the only product released from precursors in which this sequence is immediately followed by a pair of basic residues. However, the specificity constants (K_{cat}/K_m) obtained for endo-oligopeptidase A hydrolysis of bradykinin, neurotensin and dynorphin B are of the same order. Taken together, these results indicate that the substrate amino acid sequence is not the only factor determining the cleavage site of this enzyme. Finally, endo-oligopeptidase A and metalloendopeptidase EC 3.4.24.15 are two different enzymes. The latter is not able to liberate enkephalins from metorphamide and $dynorphin_{1-8}$.

Resumo: Endooligopeptidase A altamente purificada, por cromatografia de imunoafinidade, a partir da fração citosólica do cérebro de boi, foi caracterizada como uma tiol endopeptidase. Mostrou-se que esta enzima, que hidrolisa a ligação Phe^5-Ser^6 da bradicinina e a ligação Arg^8-Arg^9 da neurotensina, produz [Leu] encefalina ou [Met] encefalina por hidrólise de uma única ligação peptídica em pequenos peptídeos contendo encefalina. A formação de encefalina pode ser inibida pelo substrato alternativo bradicinina de forma dependente da concentração. Foi achado que o tamanho ótimo do seu substrato é 8-13 aminoácidos, sendo a encefalina o único produto liberado de precursores nos quais essa seqüência é imediatamente seguida por um par de resíduos básicos. Todavia, as constantes de especificidade (Kcat/Km) obtidas para a hidrólise de bradicinina, neurotensina e dinorfina B pela endooligopeptidase A são da mesma ordem. Juntos, esses resultados indicam que a seqüência de aminoácidos do substrato não é o único fator que determina o sitio de clivagen dessa enzima. Finalmente, endooligopeptidase A e metaloendopeptidase EC 3.4.24.15 são duas enzimas diferentes. A última não é capaz de liberar encefalinas de metofarmida e dinorfina 1-8.

30. TOFFOLETTO, O.; METTERS, K.M.; OLIVEIRA, E.B.; CAMARGO, A.C.M; ROSSIER, J.
Enkephalin is liberated from metorphamide and dynorphin A_{1-8} by endo-oligopeptidase A, but not by metalloendopeptidase EC 3.4.24.15.
Biochem.J., 252: 35-8, 1988.

Abstract: It has been previously reported that both the cysteinyl-endo-oligopeptidase A and the

metalloendopeptidase EC 3.4.24.15 are able to generate enkephalin from a number of enkephalin-containing peptides, including dynorphin A_{1-8}. The present study shows that only endo-oligopeptidase A is able to generate [Leu^5]enkephalin and [Met^5]enkephalin from dynorphin A_{1-8} and from metorphamide respectively. It is also shown that endo-oligopeptidase A neither hydrolyses the specific EC 3.4.24.15 substrate α-N-benzoyl-GlyAla-Ala-Phe *p*-aminobenzoate, nor is inhibited by the specific EC 3.4.24.15 inhibitor N-[1*(RS)*-carboxy-2-phenylethyl]-α-Ala-Ala-Phe-*p*-aminobenzoate.

Resumo: Foi relatado previamente que tanto a cisteinil-endo-oligopeptidase A como a metaloendopeptidase EC 3.4.24.15 são capazes de gerar encefalina de uma série de peptídeos que contém encefalina, inclusive de dinorfina A_{1-8}. O presente trabalho mostra que somente a endooligopeptidase A é capaz de gerar [Leu^5] encefalina e [Met^5] encefalina a partir de dinorfina A_{1-8} e de metorfamida, respectivamente. Ele mostra também que a endo-oligopeptidase A nem hidrolisa o substrato α N-benzoil-Gly-Ala-Ala-Phe p-aminobenzoato, específico para EC 3.4.24.15, nem é inibida por N-[1(RS)-carboxi-2-feniletil]α-Ala-Ala-Phe p-aminobenzoato, inibidor específico de EC 3.4.24.15.

31. TRONCONE, L.R.P.; FERREIRA, T.M.S.; BRAZ, S.; SILVEIRA FILHO, N.G.; TUFIK, S.
Reversal of the increase in apomorphine-induced stereotypy and aggression in REM sleep deprived rats by dopamine agonist pretreatments.
Psychopharmacology, 94: 79-83, 1988.

Abstract: REM, sleep deprivation (REMSD) induces augmented responses to dopaminergic agonits. Prolonged administration of neuroleptics induces a similar state, probably by the production of supersensitivity of dopaminergic receptors. Such a supersensitive state could be induced by REMSD as a result of impairment of dopamine neurotransmission. In order to test this hypothesis, bromocriptine, nomifensine, amphetamine, L-dopa, imipramine and electroconvulsive shock (ECS) were administered to rats during REMSD, and aggressive and stereotyped behaviors were measured. Amphetamine and L-dopa pretreatment attenuated the increases in apomorphine-induced stereotypy and aggression in REMSD rats, but ECS selectively reduced apomorphine-induced aggression. The orther drugs tested were ineffective on both behavioral tests. Such a selective action may reflect different effects of ECS on different dopaminergic systems such as those involved with stereotypy and aggression. The results suggest that REMSD induces an increase in dopaminergic sensitivity which may be reversed by pretreatment with some dopaminergic agonists.

Resumo: A privação do sono REM (PSREM) induz a um aumento da resposta comportamental a agonistas dopaminergicos. A administração prolongada de neurolépticos leva a efeitos análogos provavelmente por causar supersensibilidade de receptores, sendo que o mesmo efeito pode estar ocorrendo pela PSREM. Para se testar esta hipótese administramos bromocriptina, nomifensina, anfetamina, L-DOPA, imipramine e eletrochoque convulsivo (ECC) aos ratos durante a PSREM e avaliamos os comportamentos agressivo e estereotipado induzidos por apomorfina. Os resultados mostraram que a anfetamina e a L-DOPA reverteram o aumento observado em ambos os comportamentos enquanto que o ECC reduziu apenas a agressividade. As outras drogas foram ineficazes em alterar estes comportamentos aumentados. O efeito seletivo do ECC sobre a agressividade pode refletir a ação seletiva deste tratamento sobre sistemas diferentes de neurotransmissão. De forma global os resultados também corroboram a hipótese de supersensibilidade dopamínergica decorrente da PSREM.

32. UMEKITA, L.F.; TAKEHARA, H.A.; MOTA, I.
Role of the antibody Fc in the immune clearance of *Trypanosoma cruzi.*
Immunology Letters, 17: 85-9, 1988.

Abstract: Passive transver of immune serum obtained from mice chronically infected with *Trypanosoma cruzi* to mice containing circulating bloodstream trypomastigotes induces a very fast clearance of the parasites. Comparison of Tripomastigotes clearance in normocomplementemic and C5-deficient mice showed no difference. IgG fraction obtained from immune serum was very efficient at inducing complement-mediated lysis and immune clearance of bloodstream trypomastigotes, whereas its Fc-missing $F(ab')^2$ fragments, although able to induce lysis, were unable to induce clearance. It is suggested that the immune clearance of bloodstream trypomastigotes is dependent on the antibody Fc region and that complement-mediated lysis is not a prerequisite for elimination of the parasites from circulation.

Resumo: Transferencia passiva de soro imune obtido de camundongos com infecção crônica com *T. cruzi* para camundongos receptores com tripomastigotas na circulação resulta na rapida remoção dos parasitas da circulação. A velocidade da remoção dos parasitas da circulação foi iguai em camundongos normais ou deficientes em C5. A fração IgG obtida de soro imune foi muito eficiente para induzir lise mediada por C e a remoção dos parasitas da circulação. Os fragmentos $F(ab')^2$ obtidos da IgG mostraram-se capazes de induzir lise porém incapazes de induzir a remoção dos parasitas no sangue periferico. Sugere-se que a remoção imune dos tripomastigotas sanguíneos depende do fragmento Fc e que a lise mediada pelo C não é um pré-requisito para a remoção dos parasitas da circulação.

(1987)*

33. ÁVILA, S.C.; ROLIM ROSA, R.; IIZUKA, H.; VIEIRA, E.G.J.; FERNANDES, W.
Determinação da atividade letal do veneno de *Crotalus durissus collilineatus* (Amaral, 1926) em *Mus musculus* Linnaeus, 1758.
An.Farm.Quim. Dezembro — 1987.

Resumo: Foi determinada, em termos do DL50, a toxicidade do veneno de *Crotalus durissus collilineatus*, em camundongos, através da inoculação pelas vias intraperitoneal e intravenosa. Verificou-se que a intraperitoneal é a mais sensível à ação tóxica da referida peçonha. Por outro lado, foi também constatado que, sucessivas extrações podem provocar variação da atividade letal da mesma, além de reduzir o índice de sobrevida das serpentes.

Abstract: The lethal activity, in terms of LD50, of the venoms of *Crotalus durissus collilineatus* was determined in mice by intraperitoneal and intravenous route. It was found that both routs produce satisfactory results, however it was observed that the intraperitoneal one is more effective.
By the other hand, sucessive venom extrations may provoke a variation in the toxicity levels of the venom, besides, redution of the snakes survival rate.

34. NAKAMURA, D.; WACHTEL, S.S.; LANCE, V.; BEÇAK, W.
On the evolution of sex determination.
Proc.R.Soc.Lond. B, 232: 159-80, 1987.

Abstract: Female mice rejet skin grafts from intrastrain males because of the H-Y, transplantation antigen. Those females produce antibodies that recognize a male-specific cell-surface antigen in serological tests. The serologicaL antigen has also been called 'H-Y', but there is evidence that the two antigens are distinct. We therefore refer to the transplantation antigen as H-Yt, or transplantation H-Y, and to the serological antigen as serological H-Y, or simply H-Y, without prejudice whether these are the same or related or separate antigens.
In this study, sex-specific expression of serological H-Y antigen was found in 25 new vertebrate species representing each of seven major vertebrate classes. There was a strong correlation between expression of H-Y and occurrence of the heterogametic-type gonad, although unusual patterns of H-Y expression were noted in cases of temperature-influenced sex determination and systems representing possible transition from one mode of heterogamety to the other.
Male and female heterogamety are found side-by-side in certain freshwater toothed carps; and distinct sex chromossomes have been recognized certain amphibians, even though they are not apparent in certain reptiles and primitive birds. In seven pophidian species, in which the female is the heterogametic sex, H-Y was detected in the female; and in three species of Ranidae in which the male is heterogametic, it was detected in the male. In three species of cartilaginous fish and in one of the cyclostomes, in which heterogamety has not been ascertained, H-Y was detected in the male, suggesting that those primitive fishes are male-heterogametic. Evidently, then, heterogamety and sex-chromosome heteromorphism are polyphyletic, although certain sex-determining genes may be held in common among the diverse taxonomic groups.

Resumo: Camundongos fêmeas rejeitam transplante de pele de machos da mesma linhagem, devido ao antígeno de transplante H-Y. Essas fêmeas produzem anticorpos que reconhecem um antígeno de superfície celular, especificamente masculino, em testes sorológicos. O antígeno sorológico também foi denominado "H-Y" mas existem evidências de que os dois antígenos são distintos. Nós, portanto, nos referimos ao antígeno de transplante como H-Yt ou H-Y de transplante e ao antígeno sorológico como H-Y sorológico ou simplesmente H-Y, sem implicações de que eles sejam iguais, relacionados ou antígenos separados.
Nesse estudo, a expressão do antígeno sexual H-Y sorológico, foi encontrado em 25 novas espécies de vertebrados, representando cada uma das sete grandes classes de vertebrados. Observou-se uma forte correlação entre expressão do H-Y e ocorrência de gônada do tipo heterogamético, apesar de que padrões incomuns de expressão do H-Y foram notadas em casos de determinação do sexo influenciado pela temperatura e em sistemas representando possível transição de um modo de heterogametia ao outro.
Heterogametia masculina e feminina foi encontrada lado a lado, em certas carpas dentadas de água doce, e cromossomos sexuais distintos foram reconhecidos em certos anfíbios, apesar deles não serem aparentes em certos répteis e aves primitivas. Em, sete espécies de ofídios, nos quais a fêmea é o sexo heterogamético, o H-Y foi detectado na fêmea, e em três espécies de Ranidae nas quais o macho é heterogamético ela foi detectada no macho. Em três espécies de peixes cartilaginosos e em um de ciclostomos nos quais a heterogametia não foi confirmada, o H-Y foi detectado no macho, sugerindo que esses peixes primitivos são de heterogametia masculina. Evidentemente, portanto, a heterogametia e o heteromorfismo dos cromossomos sexuais são polifiléticos, apesar de que certos genes determinadores do sexo podem ser mantidos em comum entre os diversos grupos taxonômicos.

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* Trabalhos que não constaram do v *50*(3): 103-110, 1988.

35. RÉ, L. & KAWANO, T.
Effects of *Laurus nobilis* (Lauraceae) on *Biomphalaria glabrata* (Say, 1818).
Mem.Inst.Oswaldo Cruz, 82 (supl.4): 315-20, 1987.

Abstract: Experiments were carried out using aqueous extracts from leaves and flowers of Laurus nobilis on Biomphalaria glabrata. Treatments were performed on blastula stage (+ ou - 15h after first cleavage) and on adult snails (11-18mm). In both instances they were exposed for 24h to different concentrations of the extracts on snails (200 to 2500 ppm) and embryos (20 to 300 ppm) at 25 + ou - 1°C. The embryos were observed for a period of 20 days after treatment and the snails for 10 days.
Results obtained with leaf aqueous extracts have shown a degree of toxicity on embryos starting at a concentration of 125 ppm, the flower extract being effective at 35 ppm. The malformation obtained with the different concentrations falls into the unespecific type category, however some cephalic and shell malformations were found in embryos treated with concentrations over 50 ppm (leaves) and 25 ppm (flowers).
The LD90 on adult snails obtained by treatments with flower and leaf extract was observed at concentrations of 340 ppm and 1900 ppm respectively.

Resumo: Experimentos foram realizados utilizando-se extrato aquoso de folhas e flores de *Laurus nobilis* (louro) em *Biomphalaria glabrata*. Embriões no estágio de blástula (+ ou - 15h, após a 1ª clivagem) e adultos (11-18 mm de diâmetro) foram tratados com os extratos, durante 24 horas. As concentrações dos extratos utilizados para os caramujos foram de 200 a 2.500pp, e para os embriões foram de 200 a 300 ppm, à temperatura de 25 + ou - 1ºC. A observação diária dos embriões foi realizada durante 20 dias e para os caramujos durante 10 dias.
Resultados obtidos com embriões demonstraram que o extrato aquoso das folhas mostrou um grau de toxicidade a partir de 125 ppm, e das flores a partir de 35 ppm. A principal malformação obtida foi o tipo inespecífico encontrado nas diferentes concentrações, entretanto algumas malformações cefálica e de concha foram observadas nas concentrações acima de 50 ppm (folhas) e 25 ppm (flores) O DL90 obtido em caramujo após o tratamento com os extratos de flores e folhas foi de respectivamente 340 ppm e 1900 ppm.

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