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43. PHARMACOLOGY OF CRYSTALLINE CROTOXIN. I. TOXICITY

OSWALDO VITAL BRAZIL, JÚLIA PRADO FRANCESCHI
and EZEQUIEL WAISBICH

Department of Pharmacology, University of Campinas, Campinas, S.P., Brazil

Crotoxin is a crystallline toxin isolated for the first time in 1938 from the venom of the South American rattle-snake (*Crotalus durissus terrificus*) by Slotta and Fraenkel-Conrat (1) working at the laboratories of Butantan Institute. It behaved as a single pure protein when it was examined by E. J. Cohn's method (1), electrophoresis (2) or sedimentation and diffusion experiments in the ultracentrifuge (3). Its molecular weight was estimated to be 30.000 (3). However, as Neumann and Habermann (4, 5) have more recently shown, crotoxin can be separated into two components by column chromatography. One of these exhibits the same toxicity as crotoxin but is devoid of known enzymatic activities. It was called "crotactin" by Neumann and Habermann. The other is a phospholipase A, which according to the German authors, presents only a little toxicity. Thus crotoxin is now believed to be made up from two proteins probably united by ionic bonds.

Pharmacological data concerning crotoxin are very scarce. In fact, little more than its high toxicity for mice (6) and its stimulatory effect on the isolated guinea-pig ileum (1), was known about its pharmacological properties. Therefore, a pharmacological study of crotoxin was undertaken and will be presented in this and in the following communications.

Material and methods

The venom used in this research came from a serpentarium at Catalão, Goiás. It was a dried pool of *C. d. terrificus* venom devoid of crotamine.

Both methods devised by Slotta and Fraenkel-Conrat (1) for separating crotoxin were utilized. Nearly all batches of it were obtained in crystalline form. Crotoxin, as well as the venom was dissolved in saline immediatly before use. However, in some experiments a solution kept in the refrigerator at 4°C for no more than 7 days was also employed.

The median lethal doses of crotoxin were determined in mice, rats, pigeons, guinea-pigs and rabbits and those of the venom, in mice and rats. Four dose levels spaced in a geometric progression (common ratio 1.5) were employed. Six animals per dose were used, except in the determination of the LD_{50} for pigeons. In this case, three animals per dose were employed.

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The intravenous route was used for the determination of the median lethal doses of crotoxin and the venom for mice (17-21 g). In addition, crotoxin median lethal dose by subcutaneous route for mice was also determined. An "Agla" micrometer syringe outfit was used in this case to inject the crotoxin solution. In other determinations, crotoxin or the venom was administered by intravenous, intraperitoneal or intramuscular route. Pigeons, guinea-pigs, mice and rats were observed for 48 hours. The rabbits were discarded after the fifth day. The median lethal doses and their 95 of -100 confidence intervals were calculated by the procedure described by Weil (7).

The signs evoked by crotoxin in dogs were investigated in 36 conscious animals. The intravenous route was employed. The animals were observed for 5 to 9 days. Ten dogs, however, were anaesthetized 24 or 48 hours after crotoxin administration in order to prepare them for the study of neuromuscular transmission. To some of these animals artificial respiration was given after their spontaneous respiration had ceased. An automatic Takaoka respirator in connection with an air or oxygen cylinder was used. The urine of nearly all dogs was examined for albumin and haemoglobin. Diuresis was studied in 13 female animals which were kept for this purpose in metabolic cages for 5 or more days after crotoxin injection. In most instances, controls were performed by collecting the urines during the four days that preceded crotoxin administration.

Sixteen of the dogs were used for the determination of the crotoxin median paralysing dose. Four dose levels in a geometric progression with a common factor of 1.25 were employed. The ED_{50} was also calculated according to Weil procedure. Three unanaesthetized dogs were also intravenously injected with the venom.

The motor effects evoked by crotoxin and gallamine (Flaxedil) were compared in monkeys by using two *Cebus sp.*. Both substances were intravenously administered.

Conscious cats were injected with crotoxin by intravenous and intra (cerebro) ventricular routes. Three animals were used in both instances. Crotoxin was injected in one of the brain lateral ventricules through a Feldberg cannula inserted some days before.

Results

Crotoxin showed itself to be more toxic than the venom for both mice and rats. The ratio of their median lethal doses for these animals did not differ significantly (Table I).

Great species variability was found in the sensitiveness to crotoxin. Pigeons proved to be extremely sensitive while rats were very resistent to its lethal action (Table I). Crotoxin mean lethal dose by subcutaneous route for mice was 2.16 (1.15 to 4.02) times greater than that determined by intravenous administration. The crotoxin median paralysing dose for dogs was estimated to be 138 (106 to 179) mcg/Kg. Its mean lethal dose for this animal species was probably greater than 200 mcg/Kg (Table II).

Crotoxin given by the usual parenteral routes of administration elicited paralysis in all animal species in which it was injected. In nearly all (rats, guinea-pigs, rabbits, cats, dogs and monkeys) of them, the motor effects which began by muscular hypotonia and muscular weakness and evolved to flaccid paralysis, were identical with those observed in animals injected whith curare. The order of involvement of the various muscular groups was also the same in both

TABLE I — CROTOXIN AND *CROTALUS DURISSUS TERRIFICUS* VENOM MEDIAN LETHAL DOSES AND THEIR 95 OF 100 INTERVALS FOR VARIOUS ANIMAL SPECIES

Animal	Route of administration	Crotoxin LD_{50} (mcg/Kg)	Venom LD_{50} (mcg/Kg)	Venom LD_{50}/ Crotoxin LD_{50}
Pigeon	Intravenous	2.17 (1.35 to 3.48)	—	—
Guinea-pig	Intramuscular	38 (29 to 50)	—	—
Mouse	Intravenous	82 (66.9 to 101.4)	168.5 (135.7 to 209.85)	2.05 (1.34 to 3.14)
	Subcutaneous	177.5 (117 to 269)	—	—
Rabbit	Intravenous	110.23 (85.64 to 141.9)	—	—
Rat	Intraperitoneal	756 (564 to 1010)	1950 (1950 to 2120)	2.58 (1.77 to 3.75)

cases. The similarity of signs evoked by crotoxin and curare was very convincingly shown in monkeys (Fig. 1). Pigeons and mice were exceptions. In these animals, only large doses produced the curare-like paralysis. Small doses elicited an ascendent type of paralysis. Mice, for instance, injected with a dose of crotoxin near its LD_{50} frequently showed after a long delay, paralysis of their hind limbs while the neck and fore limb muscles were not affected.

Fig. 1 — From left to right: (1) a monkey 15 hours after receiving 100 mcg/Kg of crotoxin i.v., (2) the same monkey 30 hours later after nearly complete recovery and (3) another *Cebus sp* 3 to 5 min. after being intravenously injected with 300 mcg/Kg of gallamine (Flaxedil). Ptosis of the eyelids and of the jaw, paralysis of the muscles of the neck as well as a generalized loss of muscle strength occurred in both monkeys. They also became aphonic, could not swallow and presented diaphragmatic respiration. Consciousness in both animals seemed to be preserved.

The diaphragm was one of the last muscles to become paralysed in crotoxin intoxication. When it ceased contracting, the animals could be maintained alive under artificial respiration. This was demonstrated in cats and dogs. Crotoxin paralysis proved to be reversible in all animals except rabbits. These animals always died before recovering from the paralysis even when it was not complete.

The first signs of the motor effects caused by not too large a dose of crotoxin appeared after a long delay and the paralysis lasted for long periods of time. In dogs, for instance , the onset of loss of muscle strength generally occurred from 2 to 4 hours after the intravenous injection of 160 to 250 mcg/Kg of crotoxin and the animals became paralysed for 48, sometimes 72 hours, or even more. However, the lag between crotoxin administration and the onset of paralysis was usually a little shorter in the small laboratory animals. In addition, the duration of paralysis in the animals did not last so long.

During crotoxin paralysis consciousness and sensitivity seemed to be preserved. Paralytic dogs, for instance, shook their tails when the observer whistled or tickled them, or made efforts to move when their skin was pinched. Preservation of consciousness was also testified by the reaction that a paralytic dog presented at the incidental approach of a cat. It became furious making desperate efforts to fight with the cat.

TABLE II — SOME EFFECTS OF CROTOXIN IN DOGS

Effects	Doses of crotoxin (mcg/Kg)			
	200	160	128	102.4
Defaecation	2/4	3/4	2/4	1/4
Vomiting	3/4	2/4	0/4	1/4
Salivation	2/4	1/4	0/4	0/4
Slight muscular hypotonia (1)	4/4	4/4	4/4	4/4
Advanced muscular hypotonia and considerable loss of muscle strength (2)	4/4	2/4	3/4	2/4
Flaccid paralysis(3)	4/4	2/4	2/4	1/4
Death	1/4	0/4	1/4	0/4

(1) Some incoordination and weakness in muscular action were perceivable when the dogs were forced into locomotion.

(2) The dogs could not support themselves on their limbs because of the loss of muscular strength.

(3) The dogs lay dow helpless on one of their flanks.

Besides paralysis, other effects were observed in unanaesthetized dogs. Prior to the appearance of paralysis, one or more of the following effects frequently occurred: defaecation, vomiting, salivation (Table II). During the paralysis and after its partial or complete regression, or even in its absence, albuminuria, haemoglobinuria and oliguria or anuria (Table III) were verified. Opacity and exfoliation of the cornea as well as vomiting occurred in a few dogs after the third day of crotoxin administration. Vomiting and salivation were also observed in cats before the onset of paralysis.

TABLE III — EFFECT OF CROTOXIN ON DIURESIS

Dog (♀) Weight (Kg)	Crotoxin Dose (mcg/Kg)	Diuresis		
		Before crotoxin administration Mean of 4 days (ml/day)	After crotoxin administration Mean of 5 days (ml/day)	Per cent reduction
11.0	250	—	61.0	—
5.7	250	—	36.0 *	—
5.7	250	—	49.0	—
8.1	250	—	53.0	—
6.2	250	183.0	62.0	66.1
6.7	160	—	94.0	—
7.9	160	149.0	200.0	Increased
6.4	160	212.5	109.0	48.7
7.9	160	312.5	168.0	46.2
6.1	128	200.0	167.0	16.5
5.9	128	337.5	52.0 **	84.5
4.9	128	140.0	105.0	25.0
6.5	102.4	185.0	84.0	54.6

* Mean of 2 days.
** Mean of 4 days.

Deaths of the dogs generally took place within the first 48 hours of crotoxin injection. They were due to respiratory arrest. However, a few animals died afterwards when the paralysis was in regression. There was post-mortem evidences that in these cases shock was the cause of death. Renal lesions were demonstrated in the kidney of nearly all dogs injected with crotoxin. They will be reported in a separate communication. Hemorragic foci were never seen in the meninges or brain substance of the dogs killed by crotoxin action.

When crotoxin (50 mcg) was injected by intra (cerebro) ventricular route in cats (1 to 5.5 Kg) seizures of clonic convulsions ending in death were produced 2 to 3 hours after its administration (Fig. 2). Tachypnoea, slight clonic movements in the paws, salivation and vomiting were observed prior to the convulsive seizures.

The venom evoked signs in mice which were similar to those produced by crotoxin. In rats and dogs, however, the symptomatology differed from that elicited by crotoxin, chiefly in the latter species.

Rats injected with venom became very flaccid but never showed complete paralysis as the rats under action of crotoxin. Respiratory arrest seemed to take place before the motor effects attained that point.

The venom (250 mcg/Kg, i.v.) elicited the following effects in dogs:

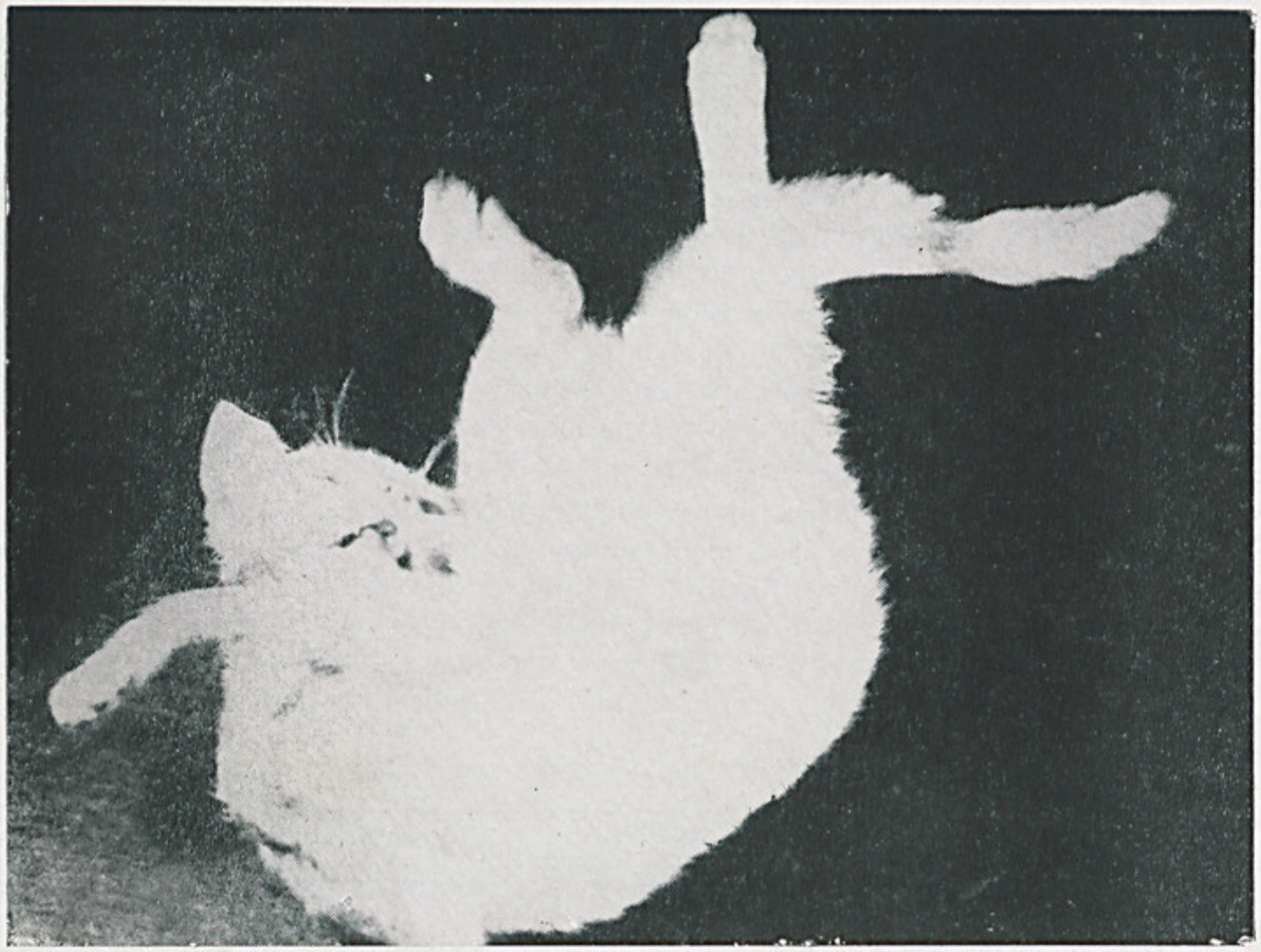

Fig. 2 — This cat (1.3 Kg) was injected with 50 mcg of crotoxin by intra (cerebro) ventricular route. The seizures of clonic convulsions (fotograph) appeared 2 hours and 20 minutes after crotoxin administration. Tachypnoea, salivation and vomiting occurred prior to the convulsive fits.

1. Immediately after its injection, tachypnoea, defaecation, micturition as well as a very short period of equilibrium loss, and nystagmus were observed. A short time later defaecation, vomiting and salivation occurred.

2. After a delay of 1 or more hours, seizures of tonic-clonic convulsions ending in death or clonic convulsive movements, usually of the limbs associated with some degree of muscular hypotonia, and loss of muscular strength happened.

All three dogs died: one of them 2 hours after the injection of the venom, another between the first and the second day and a third dog in from the third to the fourth day. Post-mortem, this last animal showed small haemorragic foci in the pia mater and zones of congestion and oedema in the lungs, besides intensily congested abdominal viscera. The mucosa of its intestines was congested and haemorragic.

Discussion

The results obtained in this research point out the following order of susceptibility to the lethal action of crotoxin: pigeons > guinea-pigs > mice > rabbits > rats (see Table I). It seems to be important, therefore, to inquire if this same order of sensitiveness occurs in relation to the venom. Our results showed

that the venom was also much more toxic for mice than for rats. Brazil and Pestana (8), on the other hand, encountered the following values for the minimal lethal doses of the venom: 1 mcg (3.3 mcg/Kg, i.v.) for pigeons, 60 mcg/Kg (i.m.) for guinea-pigs and 250 mcg/Kg (i.v.) for rabbits. It can be inferred, therefore, from these results that the order of susceptibility to the venom among pigeons, guinea-pigs, mice, rabbits and rats, is the same as that shown to crotoxin. The venom was found to be less toxic than crotoxin for mice and rats (see Table I). The figures given by Brazil and Pestana (8) refer to the minimal lethal doses of the venom and therefore are not strictly comparable with the values of the median lethal doses we determined for crotoxin. Nevertheless the analysis of their data suggests that the venom is also less toxic than crotoxin for pigeons, guinea-pigs and rabbits. As, in addition, the signs evoked by small doses of the venom and crotoxin in these animals are very close or even indistinguishable, it can be concluded that crotoxin is the component which produces the signs of envenomation and causes death in pigeons, guinea-pigs, mice and rabbits when small doses of the venom are injected. In rats which are very resistant to crotoxin (and also to the venom), other venom components participate in the envenomation, altering the symptomatology elicited by crotoxin. For dogs, the venom seems to be as toxic as crotoxin and, in addition, evokes effects, when given by intravenous route, which are very different from those produced by that substance. It gives rise, for instance, to convulsions and, sometimes, to small haemorragic foci in the pia, as first pointed out by Brazil (8, 9) and confirmed in this study. The convulsive action of the venom could be due: (a) to the penetration of crotoxin in the brain interstitial spaces after the breakdown of the blood-brain barrier produced by another component of the venom, or (b) to a toxin of the venom different from crotoxin. The first hypothesis is suggested by the convulsive action of crotoxin when introduced by intra (cerebro) ventricular route. The second one is based on the separation of a fraction from the venom which caused convulsions not only in dogs but in mice as well (unpublished data).

Several signs of systemic envenomation produced in man by the bite of the South American rattle-snake were observed in the animals injected with crotoxin, specially monkeys and dogs. Such were ptosis of the eye-lids and of the jaw, weakness of the neck and other skeletal muscles as well as general muscle paralysis. Difficulty in swallowing occurred in dogs and monkeys and is a complaint of some patients. Ptosis of the eye-lids is one of the first signs of envenomation and is probably displayed by nearly all patients bitten by *C. d. terrificus*. Vomiting within the first hours of the bite also occurs and was frequently observed in dogs injected with crotoxin (Table II). Albuminuria, haemoglobinuria, oliguria and even anuria, which were effects produced by crotoxin in dogs, are frequently observed in patients (10). However, participation of other venom components in the genesis of renal disturbances and vomiting is not excluded.

Summary

1. The toxicity of crotoxin was compared with that of the venom of *Crotalus durissus terrificus* by determining their median lethal doses for mice (i.v.) and rats (i.p.). Crotoxin showed itself to be more toxic than the venom for both animal species.

2. The median lethal doses of crotoxin for pigeons (i.v.), guinea-pigs (i.m.) and rabbits (i.v.) were also determined. The following order of susceptibility to

its lethal action was encountered: pigeons > guinea-pigs > mice > rabbits > rats. It seems that the order of sensitiveness to the venom is the same.

3. Crotoxin evoked paralysis in all animal species used. In nearly all of them the paralysis was similar to that elicited by curare in the degree of flaccidity shown by the skeletal muscles and in the order of involvement of the various muscular groups. Artificial respiration maintained the animals alive after spontaneous respiration had ceased. Consciousness and sensitivity seemed to be preserved in animals presenting crotoxin paralysis.

4. Besides paralysis, defaecation, vomiting, salivation, albuminuria, haemoglobinuria, oliguria and in some instances opacity and exfoliation of the cornea, were observed in dogs.

5. When administered by intra (cerebro) ventricular route in cats, crotoxin elicited convulsions instead of paralysis.

6. There are evidences indicating that the lethal effect of small doses of the venom in pigeons, guinea-pigs, mice, rabbits and rats is due to crotoxin. In dogs, however, it seems that death is produced by a component of the venom different from crotoxin.

7. In human beings several signs observed after the bite of *C. d. terrificus* can be assigned to crotoxin action.

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