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34. PREPARATION AND PROPERTIES OF ^{131}I-LABELLED CROTOXIN

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The *in vivo* distribution of snake venoms is still very little known. Gennaro and Ramsey (1) applied the radioisotope trace technique to get some information concerning this important aspect of venom pharmacology. They labelled the cottonmouth moccasin (*Agkistrodon piscivorus*) venom with iodine-131 and determined the radioactivity of various tissues from mice injected with this material. However, the utilization of labelled crude venom does not seem to be well suitable for this purpose. Snake venoms are complex mixtures of proteins whose molecular weight, composition in amino acids, toxicity and pharmacological actions widely differ. Suppose, for instance, the specific activities of the various components of the labelled venom under investigation are not the same. Then the radioactivity of the tissues from the animals injected with it will not give a real picture of its distribution. Moreover, the correct distribution of the more toxic components, what is really the important thing to be known, can not be clarified by using labelled crude venoms due to presence of inactive and little toxic labelled proteins in them. Therefore, it seems better to label venom components, instead of whole venoms.

A good knowledge of the pharmacology and role played in the physiopathology of envenomation of the component to be labelled is highly desirable. The labelled component may be used, in distribution studies, either alone or incorporated in the crude venom. Experiments with the last mentioned material seem to be necessary as some venom components may alter the rate of absorption of other venom components and the biological barriers of the organism, thus affecting their distribution.

An investigation on distribution, points of fixation in tissues at cellular and molecular levels, rate and pathways of absorption and excretion of snake venoms has been planned according to the criteria outlined. The venom of the Southamerican rattlesnake (*Crotalus durissus terrificus*) was selected for this study and the labelling of crystalline crotoxin, its main toxin, with radioiodine, tried. The method of iodination used to label crotoxin as well as the physicochemical and biological characteristics of the radiocrotoxin obtained are reported in this paper.

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Material and methods

Crotoxin — Crystalline crotoxin was prepared according to the methods developed by Slotta and Fraenkel-Conrat (2). Solutions for iodination were made at the time of the experiments by dissolving 30 mg of crotoxin in 10 ml of saline. The pH of the solutions, originally between 4.5 and 5.0 was adjusted to 8.0.

Counting — A NaI (Tl) 3″ × 3″ well-type scintillation counter was used with the pulse-height analyzer set at the ^{131}I keV photopeak. Counts were accumulated during a time sufficient to achieve a statistical error not greater than 3%.

[^{131}I] iodide — Sodium [^{131}I] iodide, carrier-free, was supplied by the Radiochemistry Division of the Atomic Energy Institute, São Paulo, in a strongly alcaline solution (pH 12.0). The radioiodine used was free of reducing agents.

Iodination — McFarlane's (3) method was used. Pre-oxidation of the protein as suggested by McFarlane was employed in two preliminary experiments. It was carried out with a solution of iodine-iodide at pH 4.5 followed by filtration of the mixture through a IR-4B Amberlite column in order to remove the excess of iodine. The pH of the eluates was adjusted to 8.0 or 9.0. No pre-oxidation was used in the 12 subsequent experiments.

After iodination, unbound radioiodine was usually remove from the mixture by an ion exchange resin (Amberlite IR-4B). In one of the experiments, part of the mixture was submitted, for this purpose, to dialysis against running water for 24 hours at room temperature.

The adopted procedure to label crotoxin with iodine-131 was as follows. The pH of a sodium [^{131}I] iodide solution with total activity from 3 to 8mCi was adjusted to pH 8.0 or 9.0 with hydrochloric acid and buffered with alcaline glycin (pH 8.5). Then, 0.7 ml of a ^{127}I Cl solution containing 2.94 mg of iodine were added in order to obtain the ^{131}I Cl (Iodine-131 monochloride) by isotopic exchange. The crotoxin solution was jet sprayed over this "iodination mixture", and after strong agitation and 2 to 4 minutes of rest, passed through the anionic resin Amberlite IR-4B. The total activity was then determined.

Physicochemical assays — The radiochemical purity of the iodinated material was investigated by thin layer chromatography (silica gel G, 250 u thick), a mixture of acetone, n-butanol, concentrated amonia and water (65:20:10:5) being used as solvent. The running time was 25 minutes. Crotoxin which remained at the origin (Rf = 0) was developed by nihidrin, and iodine, that migrated with the solvent (Rf = 0.7), by lead acetate. After drying, the plates were divided in three parts and counted. Unbound iodine in the ^{131}I-labelled crotoxin solution was also estimated by paper electrophoresis (Whatman n.° 1). It was performed with barbital-sodium barbital buffer pH = 8.6, $\mu = 0.05$ and 2 mA per strip for 50 minutes. The labelled crotoxin did not migrate (Rf = 0).

Iodination of the crotoxin molecules was verified by the trichloroacetic acid test. The protein was precipitate by adding the acid to the mixture, the precipitate obtained being washed several times and its radioactivity finally determined.

Labelled crotoxin was compared with the original crotoxin by means of paper electrophoresis. The buffer, ionic strength and pH were as referred. Running time of 4 and 18 hours were used. The electrophoretic strips containing the radiocrotoxin were also analyzed in the "Vanguard radioscanner".

Protein determination — Protein (crotoxin) concentration in the Amberlite eluated was estimated by determining its nitrogen content. The micro-Kjeldahl and the ultra-violet (260 mμ and 280 mμ) differential spectrophotometry methods were used. The nitrogen content of the original crotoxin was also determined.

Biological assays — The toxicity of different batches of labelled crotoxin was compared with that of the original ones by determining their median lethal doses for piedgeons and mice. Four dose levels in geometrical progression (common factor 1.5) and six or exceptionally ten animals per dose were used. The animals were observed for a period of 24 hours. The median lethal doses and their 95 of 100 confidence intervals were calculated by Weil procedure (4).

The method of Cesari and Boquet (5) was employed for evaluating the haemolytic (phospholipasic) activity of the labelled and unlabelled crotoxin preparations. Inactivated sera and blood cells from dogs were used in these assays.

Four conscious dogs were intravenously injected with 0.25 mg/Kg of the radioiodinated crotoxin. Three of them were prepared as described in a previous paper (6), for recording contractions of the tibialis anterior muscle and arterial blood pressure, after being anaesthetized with sodium pentobarbital (20 mg/Kg, i.v.). Two dogs were prepared 24 and one 48 hours after the injection of the labelled crotoxin. Stimulation of the peroneal nerve was carried out with supramaximal square wave pulses of 0.2 msec and 0.1 c/sec. When stimuli up to 100 v did not elicit muscle responses, it was assumed that neuromuscular transmission was completely blocked. Direct muscle stimulation was done with shocks of 50-100 v, 2 msec and 0.1 c/sec.

One of the four dogs was sacrificed nine days after the administration of the radioiodinated crotoxin. The kidneys of this dog and those from the animal which was used for studying neuromuscular transmission 48 hours after radioiodinated crotoxin administration were removed for histopathological examination. The urines from these dogs were examined for albumin and haemoglobin.

The action of the labelled crotoxin was compared with that of the original one on the isolated guinea-pig ileum. The intestine was suspended in an organ bath with Tyrode solution at 37°C. A frontal inscription isotonic lever was employed. The preparation was oxygenated with air.

Results

A good labelling efficiency did not occur when pre-oxidation was performed. In the remaining experiments, a yield of 35 to 40 per cent of the initial radioactivity was obtained. The specific activity of the radioiodinated crotoxin was in this case always greater than 120 μCi/mg.

The evaluation of unbound iodine by chromatographic and electrophoretic methods gave identical results. There was from 2 to 6 per cent of free ^{131}I in the different preparations. On the other hand, the trichloroacetic acid test showed that from 94 to 98 per cent of the total radioactivity were present in the precipitates.

No differences were detected in the electrophoretic behaviour of crotoxin and radiocrotoxin (Fig. 1). Analysis of the electrophoretic strips of labelled crotoxin in the radioscanner revealed the presence of two peaks (Fig. 1)..

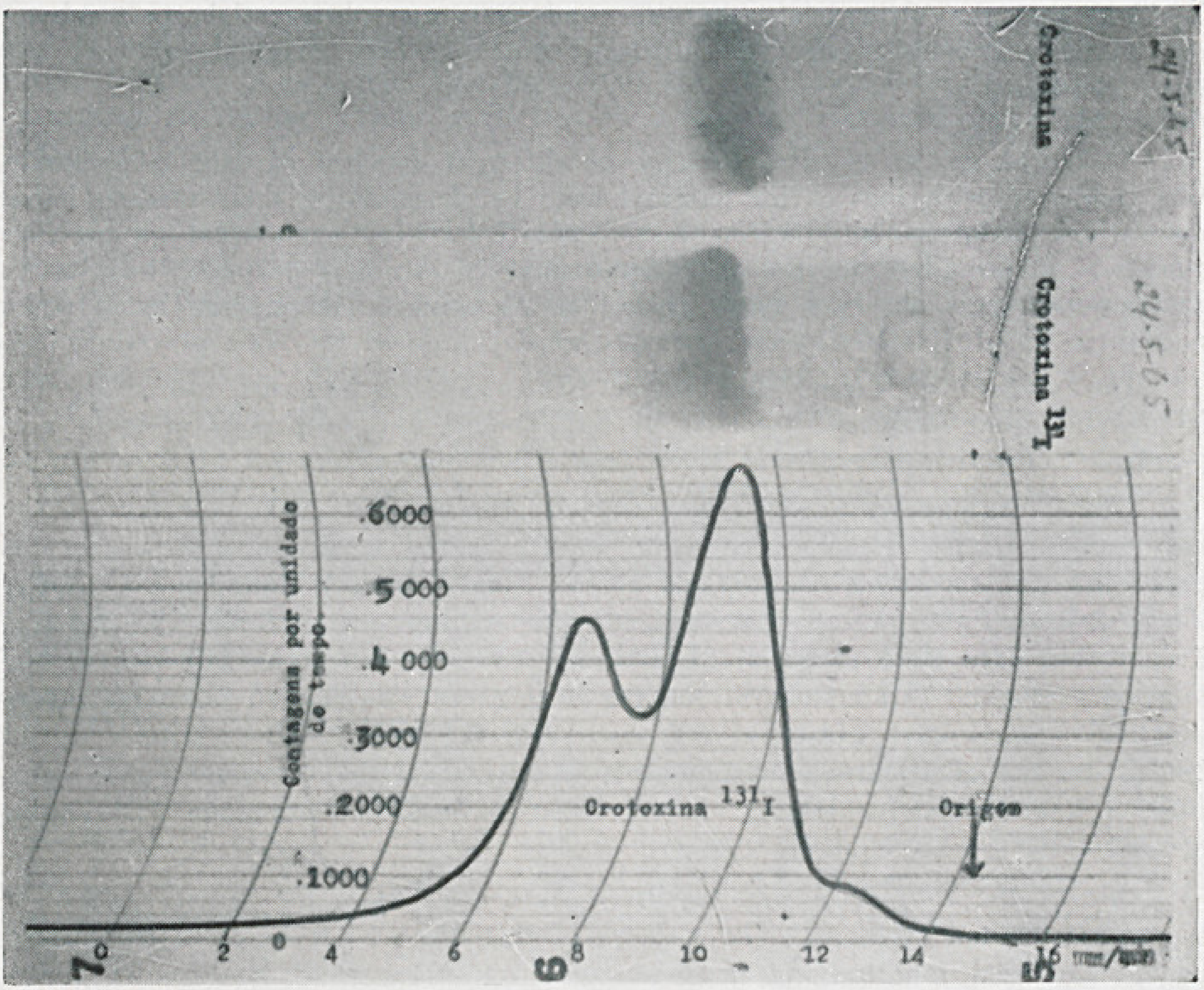

Fig. 1 — Top — Crotoxin and radiocrotoxin electrophoresis (pH 8.6, ionic strenght 0.05, 2 mA, veronal-sodium veronal buffer) strips. Bottom — Radioscanner of the labelled crotoxin.

The toxicity of radioiodinated crotoxin and that of crotoxin for pidgeons and for mice did not differ significantly. The median lethal dose, for instance, of a batch of crotoxin for pidgeons was 3.15 (1.62 to 4.18) mcg per Kg; after iodination, its LD_{50} was found to be 3.44 (3.40 to 3.45) mcg per Kg.

Table I and figure 1 show the median lethal doses of different batches of crotoxin and radioiodinated crotoxin for mice. The symptoms presented by the animals injected with crotoxin and radiocrotoxin were the same.

The haemolytic activity of the labelled crotoxin preparation which were passed through the anionic resin resulted somewhat lower than that of the original crotoxin. However, when the free iodine was removed by dialysis, the haemolytic activity of both preparations was the same (Table II).

TABLE I — TOXICITY FOR MICE (18 TO 21 g) OF SOME LABELLED AND ORIGINAL CROTOXIN PREPARATIONS

Doses in micro-grams	Results (deaths in 24 hours)				
	Experiment I (07/08/65)		Experiment II (18/09/65)		Experiment III (16/11/65)
	Crotoxin	Radiocrotoxin	Crotoxin	Radiocrotoxin	Radiocrotoxin
0.66	—	—	0/6	0/6	0/6
1.00	0/6	0/6	1/6	2/6	2/6
1.50	0/6	0/6	4/6	5/6	5/6
2.25	5/6	5/6	6/6	6/6	6/6
3.37	6/6	6/6	—	—	—

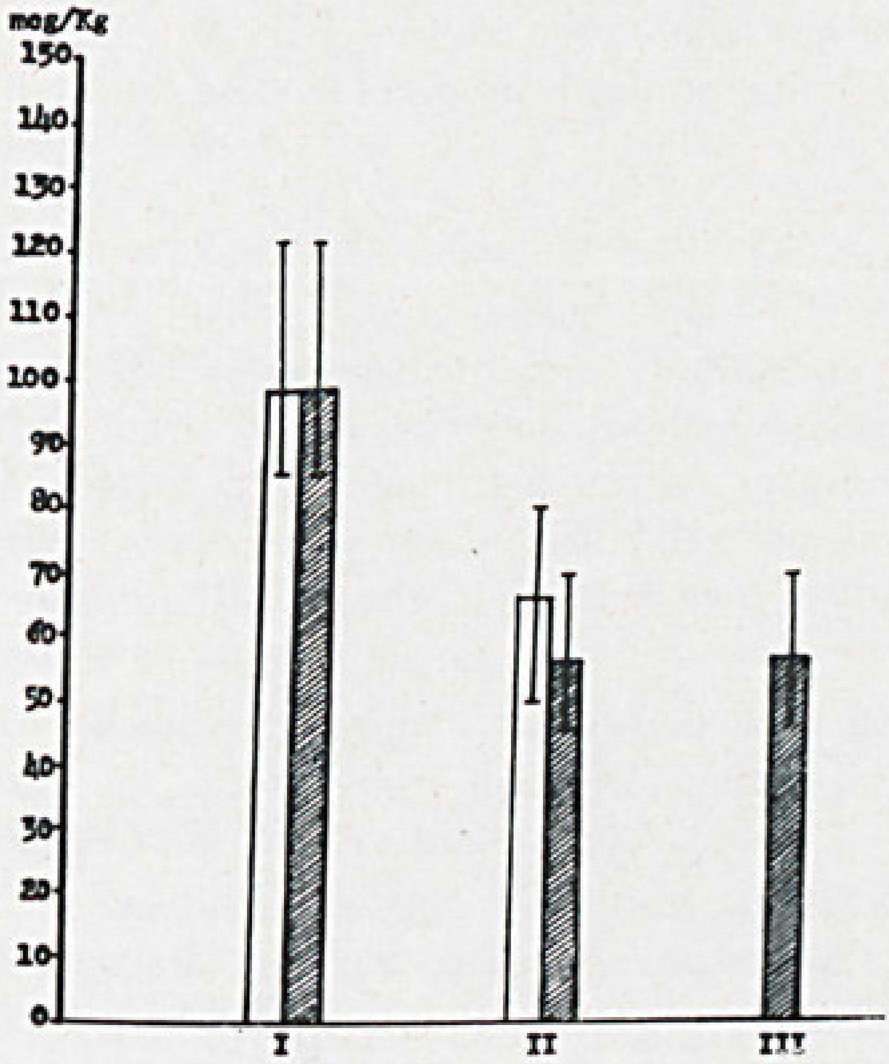

Fig. 2 — Mean lethal doses (mcg/Kg) and their 95 of 100 confidence intervals of the same preparations referred to in Table I.

Vomits, defaecation, long lasting flacid paralysis, albuminuria and haemoglobinuria were the symptoms presented by the dogs injected with the radioiodinated crotoxin. They were identical to those showed by dogs injected with crotoxin (7).

Partial or complete neuromuscular blockade (Fig. 3) was demonstrated in the dogs injected with radiocrotoxin. Therefore, neuromuscular action was identical to that of crotoxin (6).

The renal lesions found in the dogs injected with crotoxin (8) were also observed in the kidneys of the dogs intoxicated by ^{131}I-crotoxin.

The labelled crotoxin contracted the isolated guinea-pig ileus as crotoxin does (9). There was cross dissensitization between crotoxin and radioiodinated crotoxin.

TABLE II — *IN VITRO* HAEMOLYTIC ACTIVITIES OF CROTOXIN AND RADIOIODINATED CROTOXIN

Doses in micrograms	Crotoxin	^{131}I Crotoxin	^{131}I Crotoxin *
10	+++	+++	+++
5	++	+	++
2	+	0	+

* Dialysed radiocrotoxin.

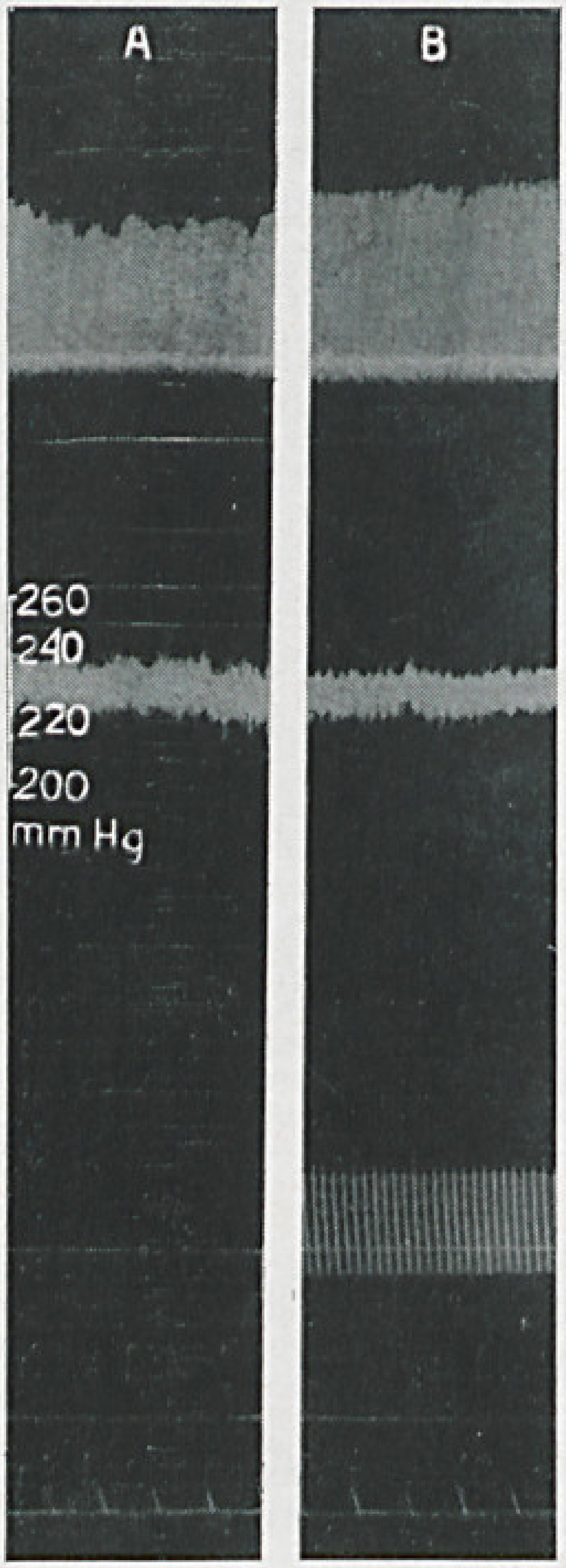

Fig. 3 — Records of the respiratory movements, blood pressure, and of the tibialis anterior muscle contractions of a dog (18 Kg, pentobarbital anaestesia), injected 22 hours before with 250 mcg/Kg of radiocrotoxin. A — Stimulation of peroneal nerve with square wave pulses of 0.2 msec., 0.1 c/sec and 100 v. B — Direct mucle stimulation with pulse of 2 msec, 0.1 c/sec and 100 v.

Discussion

The results provided by the trichloroacetic acid tests and by the analysis of the electrophoretic strips in the radioscanner showed that there was a real iodination of crotoxin, a protein or protein complex with a high aromatic amino acid content (9). On the other hand, the specific activity obtained by the method of iodination used is sufficient for crotoxin distribution studies.

Iodination did not induced important physicochemical modifications in the crotoxin molecule. This can be inferred from the results of the biochemical and biological assays. The smaller haemolytic activity of the radiocrotoxin was not due to iodination. It could be traced to the passage through the anionic resin: A radioiodinated preparation submitted to dialysis instead of being passed through the resin, presented the same haemolytic activity as the original crotoxin.

Crystalline crotoxin behaves as a homogeneous protein with a molecular weight of the order of 30.000 in sedimentation and diffusion experiments carried out with the ultracentrifuge (10). It behaves also as a uniform protein when examined by Cohn method (2). Signs of nonuniqueness of crotoxin have not been observed by mean of electrophoresis either (11). However, in the present investigation it was shown by the analysis of the electrophoretic strips of radio-iodinated crotoxin in the radioscanner the presence of two peaks of maxinal radioactivity. This seems to demonstrate that electrophoresis can start a separation of crotoxin in two components, a fact not revealed by the common procedures. It is relevant to remember that, according to Neuman and Habermann (12, 13) crotoxin is made up of two proteins: A phospholipase A of low toxicity and a toxin of high toxicity ("crotactin") which are probably linked by ionic bounds.

Summary

McFarlane's method of iodination was used for labelling crystalline crotoxin, the main toxin of the South American rattlesnake venom, with radioiodine. Yields of 35-40 per cent of the original radioactivity were obtained. Free iodine was removed by means of filtration through an anionic resin (IR-4B Amberlite) column. Radioiodinated crotoxin with specific activities greater than 120 μCi per gm was obtained.

Radioiodination of crotoxin was demonstrated by the trichloroacetic acid test and by submitting the electrophoretic strips of the iodinated preparations to analysis in the radioscanner.

Biochemical and biological assays of ^{131}I-labelled crotoxin revealed that the iodination did not induced important physicochemical modifications in the crotoxin molecule.

In view of the results of the present investigation, the authors suggest that the ^{131}I-labelled crotoxin is a convenient tool for the investigation of the distribution and rates of absorption and excretion of crotoxin as well as its points of fixation in tissues at cellular and molecular levels.

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