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# 44. PHARMACOLOGY OF CRYSTALLINE CROTOXIN. II. NEUROMUSCULAR BLOCKING ACTION

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In the precedent communication (1), it was shown that the motor signs observed in animals injected with crotoxin are very close to those evoked by curare. This suggested a peripheral origin for the paralysis caused by crotoxin and incited us to investigate neuromuscular transmission in animals to which this substance was administered. In the present paper, evidence is given indicating that block of neuromuscular transmission is really the cause of the paralysis. Some characteristics of the neuromuscular blockade elicited by crotoxin was also investigated as well as ganglionic transmission in animals injected with this venon component. In addition the effect it produces on the sensitiveness of striated muscle to acetylcholine or potassium was studied on the isolated and cronically denervated rat hemi-diaphragm.

## Material and methods

In order to decide if neuromuscular transmission is blocked or not in the animals showing the paralysis evoked by crotoxin, 10 conscious dogs were intravenously injected with 0.2 to 0.5 mg/Kg of this substance. Twenty-four or forty-eight hours thereafter, they were anaesthetized with sodium pentobarbital (20 mg/Kg, i.v.) and prepared as follows. A drill was inserted in the superior third of the tibia of one of the legs and attached through a special adjustable block to a upright fitted on a iron base plate in order to immobilize the limb of the animal. The peroneal nerve was exposed in the upper antero-lateral aspect of the leg and sectioned. To its distal end platinum electrodes were attached. The tendon of the tibialis anterior muscle was then exposed, sectioned, and connected by means of a strong thread to a flat spring steel myograph of a Brown-Schuster myograph apparatus. Two needle electrodes were inserted in the muscle, one in its belly, the other near the tendon. The trachea was exposed and cannulated. A tracheal cannula provided with two upper lateral outlets was used. In most experiments one of these was put in connection with a Marey tambour in order to record the respiratory movements of the animal. A François Franck cannula was then inserted in one of the carotids for recording the arterial blood pressure through a Ludwig's manometer. A polyethylene cannula was inserted in the femoral vein of the opposite side to the prepared limb for intravenous administration of drugs.

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Nerve stimulation was carried out with supramaximal shocks of 0.2 msec duration delivered at a rate of 30 per minute. When the muscle did not respond to stimuli of up to 100 v. applied to the nerve, it was assumed that transmission was completely blocked. Stimuli of a higher (50 c/s) frequency were also employed for short (10 to 40 seconds) periods of stimulation. Direct muscle stimulation was made with shocks of 2 msec. duration and 50 to 100 v. The stimuli used were square wave pulses from a Grass S 4 Stimulator.

Neostigmine methylsulfate (0.1 mg/Kg) and calcium chloride (0.5 ml/Kg of a 10% solution, as well as a 10% solution of sodium bicarbonate, were intravenously injected in some dogs.

The neuromuscular blocking action of crotoxin was also studied in cats. Sixteen animals were anaesthetized with sodium pentobarbital (30 mg/Kg, i.v.) and prepared as described for dogs. Nerve stimulation was made with supramaximal pulses of 0.2 msec duration delivered at a rate of 6 per minute. Shocks of 2 msec duration and 50 to 100 v. were used for directly stimulating the muscle. Large doses (0.5 mg/Kg and 1 mg/Kg, i.v.) of crotoxin were used in order to obtain neuromuscular blockade within the first three hours of its administration. The effect produced by neostigmine methylsulfate (0.1 mg/Kg, i.v.), edrophonium chloride (0.5 mg/Kg, i.v.) and succinylcholine chloride (0.02 or 0.05 mg/Kg, i.v.) on the neuromuscular blockade was investigated.

Ganglionic transmission was studied in four of the cats which were utilized in the investigation of the neuromuscular action of crotoxin. The vagus-sympathetic trunk was exposed and sectioned. In two cats, the sympathetic chain was, in addition, separated from the vagus nerve. Platinum electrodes were then attached to the upper end of either the sympathetic chain or the whole vagus-sympathetic trunk. The head of the animal was firmly fixed by a cat holder and the nictitating membrane connected with a Starling heart lever. Pulses of 0.2 msec duration and 2 to 5 v. delivered at a rate of 5 per second were used. Arterial blood pressure was also recorded.

Artificial respiration was employed whenever necessary.

Crotoxin-acetylcholine and crotoxin-potassium antagonism were investigated on the isolated and cronically denervated rat hemidiaphragm preparation. The left phrenic nerve of rats was sectioned under ether anaesthesia as described in a previous paper (2). After 25 to 35 days, the rats were anaesthetized with chloral hydrate (300 mg/Kg, i.p.) and bled. The denervated hemi-diaphragm was then removed and suspended in a organ bath containing 8 ml of Tyrode solution with 0.2 per cent glucose at 37°C. The preparation was aerated with a mixture of 95% $O_2$ and 5% $CO_2$. The contractures were recorded with an isotonic lever. Logarithmically spaced doses (common ratio 2) of acetylcholine or potassium chloride were added to the bath and allowed to act for 30 to 40 seconds. After each addition of acetylcholine or potassium chloride the bath fluid was changed four times and the preparation left for 10 minutes. The following doses of crotoxin were used in the experiments with acetylcholine: $0.5 \times 10^{-6}$, $10^{-6}$, $2 \times 10^{-6}$ $4 \times 10^{-6}$. In the experiments with potassium, only one dose ($2 \times 10{-}^{6}$) of crotoxin was employed. All contractures were expressed as percentages of the contracture obtained by immersing the preparations in a 0.1M $K_2SO_4$ solution.

The crotoxin used in this investigation was prepared as indicated in the precedent paper (1).

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## Results

Block of neuromuscular transmission was demonstrated in all of the 10 dogs which had been injected 24 or 48 hours before the experiment with crotoxin (Table I). The blockade was complete in 4 of them and partial (Fig. 1) in the remaining. Neostigmine, calcium chloride and sodium bicarbonate did not remove the neuromuscular blockade nor ameliorate the transmission. A partial recovery of muscle responses or a great increase of them always appeared after

TABLE I — NEUROMUSCULAR TRANSMISSION * AND ARTERIAL BLOOD PRESSURE OF DOGS 24 OR 48 HOURS AFTER INTRAVENOUS INJECTION OF CROTOXIN

| Crotoxin dose (mcg/Kg) | Hours after injection | Neuromuscular blockade | Arterial blood pressure (mm Hg) |
|---|---|---|---|
| 500 | 24 | complete | 170 |
| 500 | 24 | partial | 120 |
| 500 | 48 | partial | 116 |
| 250 | 24 | complete | 104 |
| 250 | 24 | partial | 220 |
| 250 | 24 | complete | 84 |
| 250 | 48 | partial | — |
| 250 | 24 | complete | 230 |
| 200 | 24 | partial | 186 |
| 200 | 24 | partial | 160 |

* Sciatic nerve-tibialis anterior muscle preparation.

tetanic stimuli were applied to the nerve (post-tetanic facilitation, Fig. 1). Cessation of the spontaneous respiration occurred in three dogs in early stages of the experiments; artificial respiration secured their survive. All dogs showed flaccid paralysis and their respiration was diaphragmatic before being anaesthetized.

In the cat experiments, crotoxin produced complete or partial neuromuscular blockade within the first 3 hours of its administration. Inhibition was complete at that time in 3 out of the 9 animals injected with 0.5 mg/Kg and in 5 out of the 7 to which 1 mg/Kg of crotoxin was administered. Neostigmine was without effect on the neuromuscular block. Edrophonium increased the partially inhibited responses of the tibialis anterior muscle. However, the effect was short-lived (Fig. 2). Succinylcholine also increased the twitches in 6 out of 8 experiments (Fig. 3). This effect was produced by doses of the depolarizing drug which caused partial blockade before crotoxin administration (Fig. 3).

The stimulation of the cervical sympathetic trunk elicited contractions of the nictitating membrane in all four cats. In two animals, the contractions attained a very good tension (Fig. 4). These cats had been injected with 0.5

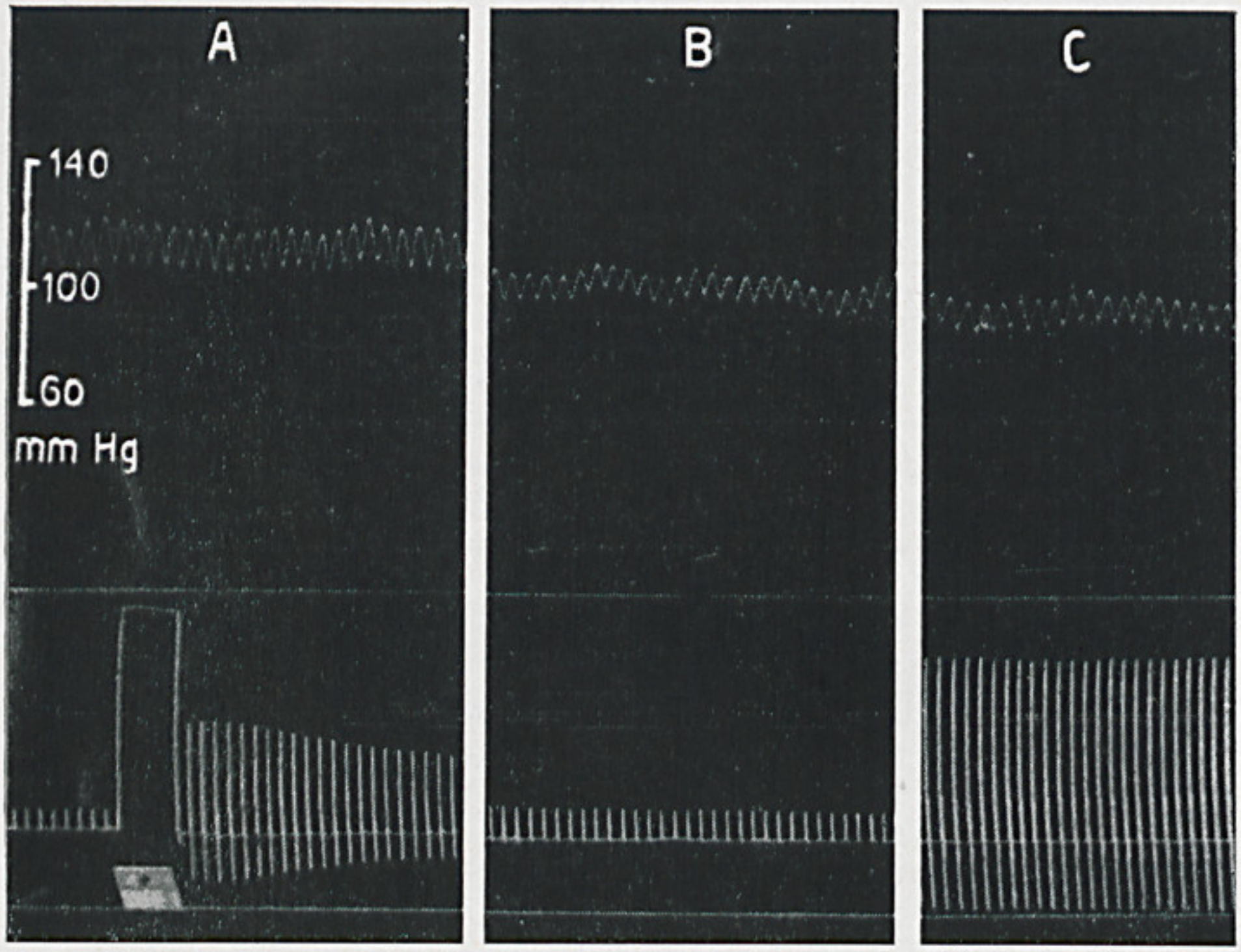


Fig. 1 — Dog, 7 Kg, sodium pentobarbital. Records of arterial blood pressure and contractions of tibialis anterior muscle. The peroneal nerve was stimulated at A and B with supramaximal shocks of 0.2 msec duration delivered at a rate of 30 per minute; at signals, shocks of a high (50 cycles/second) frequency were applied to the nerve for 10 seconds. At C, direct stimulation (pulses of 100 v. and 2 msec duration delivered at a rate of 30 per minute) of the tibialis anterior muscle. This animal had been intravenously injected 48 hours before the experiment with 0.25 mg/Kg of crotoxin. It was paralytic when anesthetized with 20 mg/Kg of sodium pentobarbital to be prepared for recording arterial blood pressure and the contractions of the tibialis anterior muscle.

mg/Kg of crotoxin. In the other two, the contractions were somewhat weaker but had no tendency to decrease in tension during the experiments. Therefore, it seems that there was also no ganglionic blockade in these animals which had been injected with 1 mg/Kg of crotoxin. In all four cats, the responses of the tibialis anterior muscle were already blocked and in three of them, the spontaneous respiration, in addition, had ceased before the beginning of the experiments.

Crotoxin produced a shift of the dose-response curve for acetylcholine to the right. A slight decline of the slopes of the curves occurred (Fig. 5). The inhibitory effect produced by crotoxin on the acetylcholine contractures could not be removed by washing repeatedly the preparation. However, some decrease of it was obtained. The dose-response curve for potassium (Fig. 6) was slightly shifted to the left by $2 \times 10^{-6}$ of crotoxin. The contractures elicited by acetylcholine or potassium after treatment of the muscle with crotoxin were not so sustained as before.

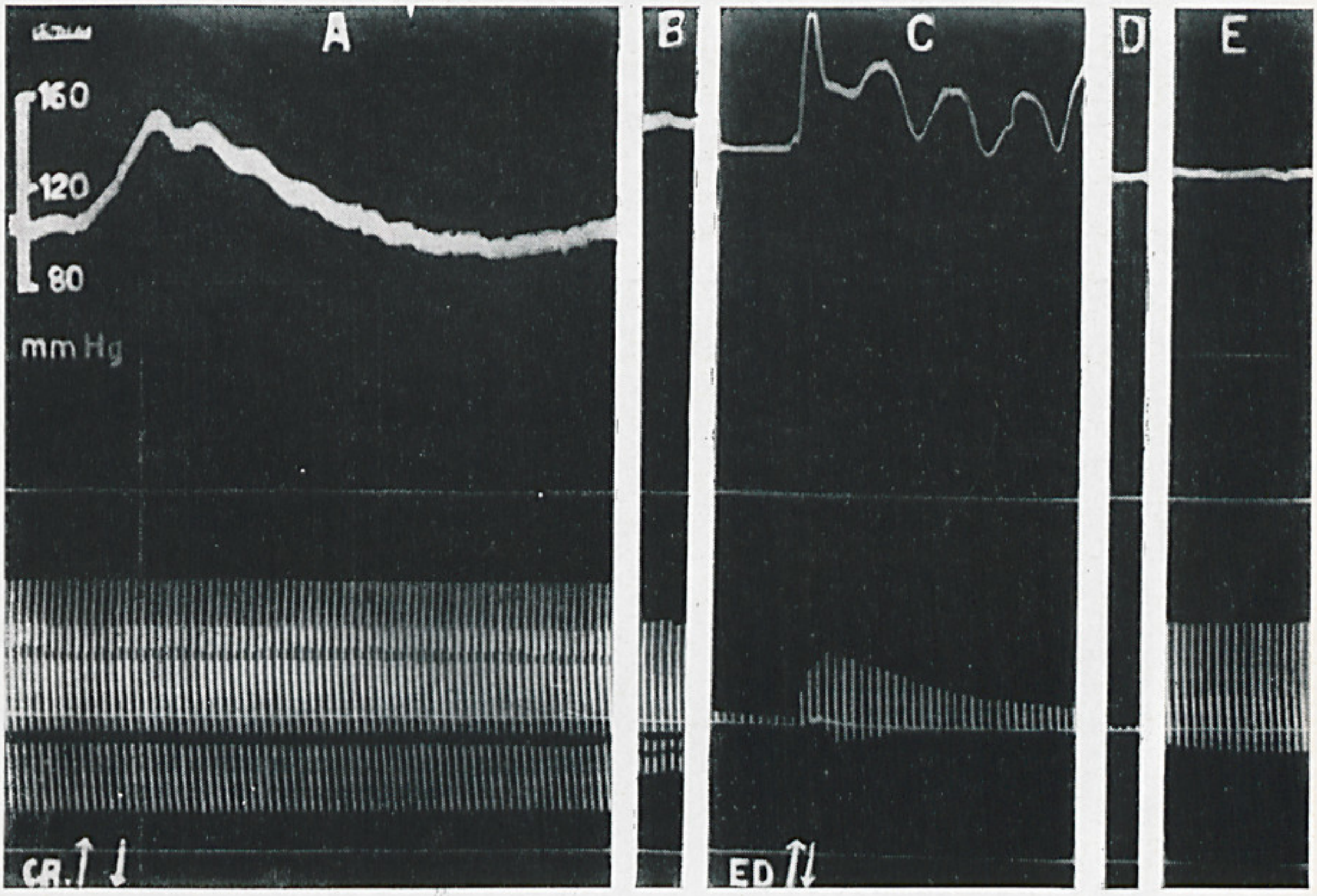


Fig. 2 — Cat, 4 Kg, sodium pentobarbital. Records of arterial blood pressure and contractions of the tibialis anterior muscle. The peroneal nerve was continuously stimulated with supramaximal shocks of 0.2 msec duration delivered at a rate of 6 per minute. At E, direct stimulation (shocks of 100 v., 2 msec timed 10 seconds apart) of the muscle was done. At Cr., 1 mg/Kg of crotoxin and at ED, 0.5 mg/Kg of edrophonium were intravenously injected. The records at B, C, D, and E were taken 70, 105, 135 and 150 minutes after A.

## Discussion

This study showed that the neuromuscular blockade produced by crotoxin can per se explain the paralysis observed in the animals injected with it. This does not mean, of course, that an action of crotoxin on the central nervous system does not contribute to the paralytic effect. However, the following results do not seen to support this hypothesis. First, the experiments concerning the effects evoked by the intra (cerebro) ventricular administration of crotoxin (1) pointed out that this substance exerts a stimulating action, not a depressing one, if it gains access to the brain interstitial fluid. Secondly, consciousness and sensibility seemed to be preserved in the animals showing the paralysis evoked by crotoxin. Thirdly, the order of involvement of the various muscular groups during crotoxin intoxication was similar to that elicited by drugs which act peripherally, and differed, therefore, from that produced by compounds like mephenesin whose loci of action are in the central nervous system. Therefore,

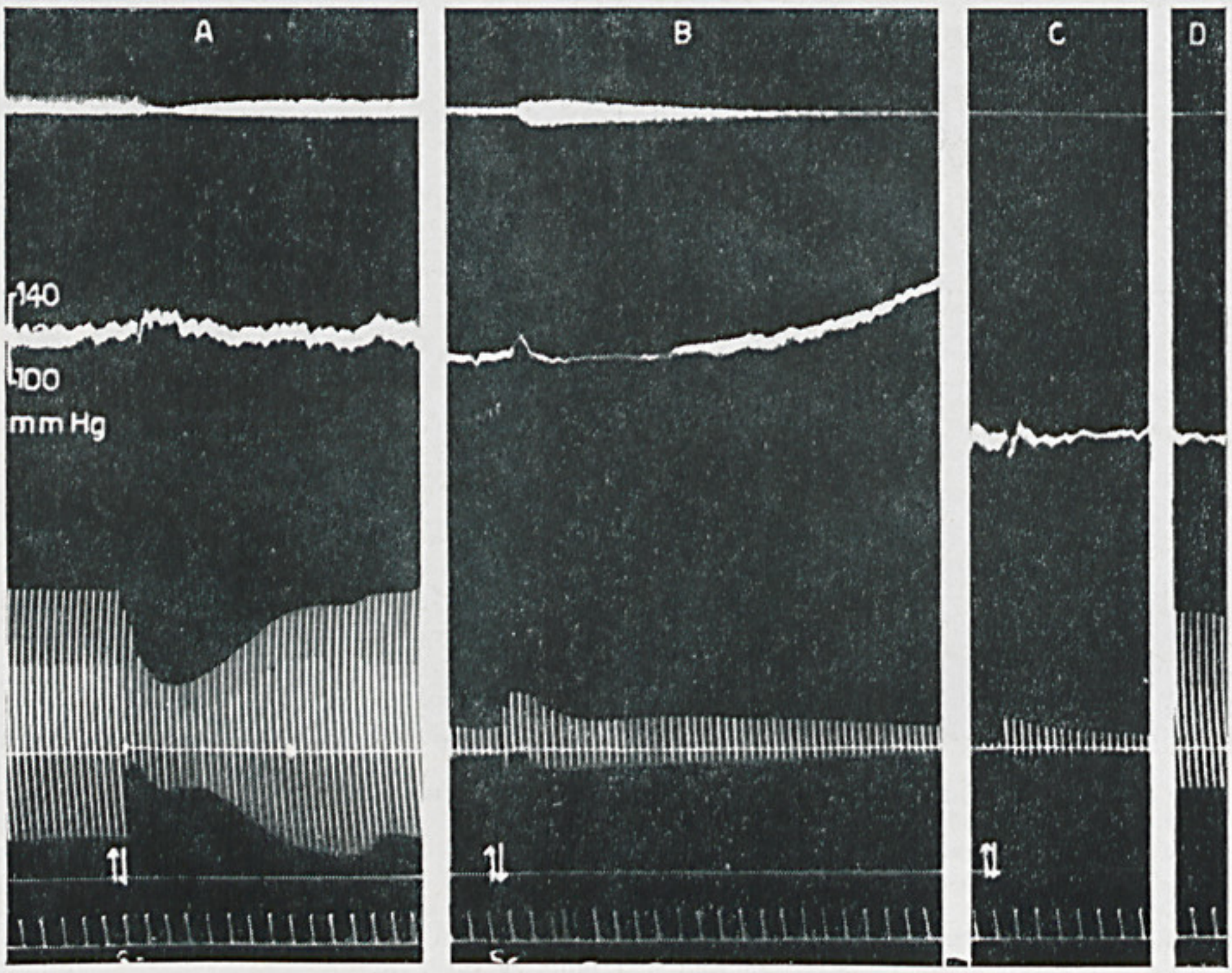


Fig. 3 — Cat, 2.4 Kg, sodium pentobarbital. Records of respiration, arterial blood pressure and the contractions of the tibialis anterior muscle. The peroneal nerve was continuously stimulated with supramaximal shocks of 0.2 msec duration delivered at a rate of 6 per minute. Direct stimulation of the muscle (at D) was done with pulses of 100 v.,, 2 msec timed 10 seconds apart, after complete neuromuscular block had occurred. At A, 0.05 mg/Kg of succinylcholine was intravenously injected before crotoxin administration; at B and C, the same dose of the depolarizing drug was injected 105 and 122 minutes after the intravenous administration of 1 mg/Kg of crotoxin. Artificial respiration (at C and D) was given after the spontaneous respiration had ceased.

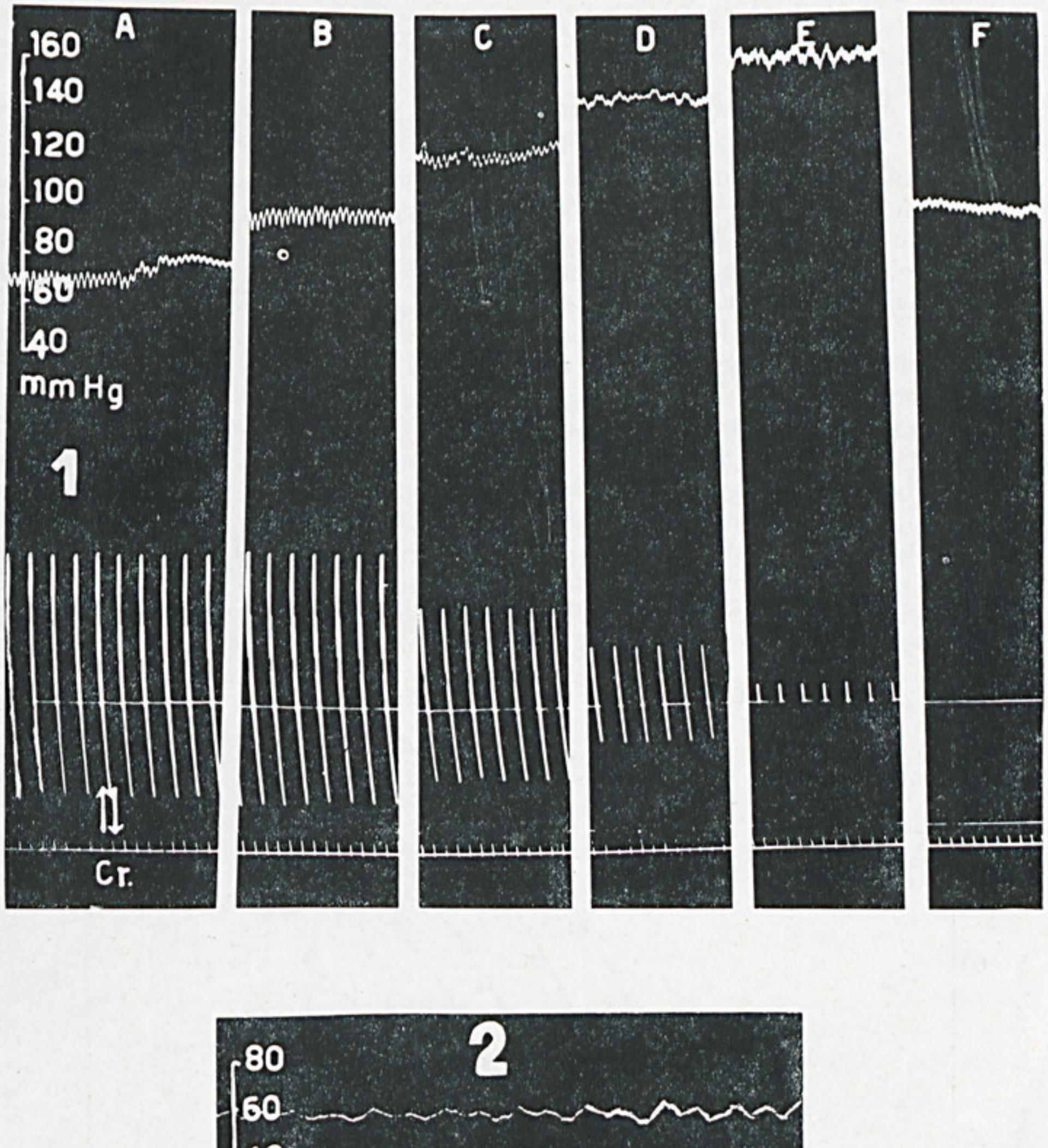


Fig. 4 — Cat, 2.5 Kg, sodium pentobarbital. 1. Records of arterial blood pressure and contractions of the tibialis anterior muscle. The peroneal nerve was stimulated with supramaximal shocks delivered at a rate of 6 per minute. B, C, D, E and F are records taken 30, 60, 90, 120 and 150 minutes after the intravenous administration of 0.5 mg/Kg of crotoxin. 2. Records of arterial blood pressure and contractions of the nictitating membrane. The cervical sympathetic chain was stimulated with shocks of 2v, 0.2 msec, delivered at a rate of 5 per second; the stimulations were done for 15 seconds periods followed by periods of 1 second rest. The records of the contractions of the nictitating membrane were taken nearly 4 hours after the administration of crotoxin.

it can be concluded that in all probability the paralysis caused by crotoxin is exclusively due to an interruption of neuromuscular transmission.

The blockade produced by crotoxin is of the non-depolarization type as can be inferred from a comparison of the results obtained in this research with those given by the study of the depolarizing drugs (3, 4). Edrophonium and succinylcholine, for instance, did not deepen the blockade as they would do if it was caused by a persistent depolarization of the end-plate region of the muscle fibres. Instead, both drugs increased although only to a small degree the partially blocked response of the cat tibialis anterior muscle. Furthermore, during crotoxin neuromuscular block, tetanization of the motor nerve was followed by a post-tetanic partial relief of it, a result which also would not have occurred if transmission was interrupted by a depolarization blockade. The same conclusion can be drawn from the absence of muscle fasciculations in cats and other animals injected with crotoxin as well as from the lack of any stimulating action of this substance on the denervated rat hemi-diaphragm. Depolarizing drugs in birds give rise to contracture of skeletal muscles, not to flaccid paralysis. Small doses of crotoxin did not elicited, as previously reported(1), a flaccid paralysis of the curare type in pigeons. However, the symptoms presented by

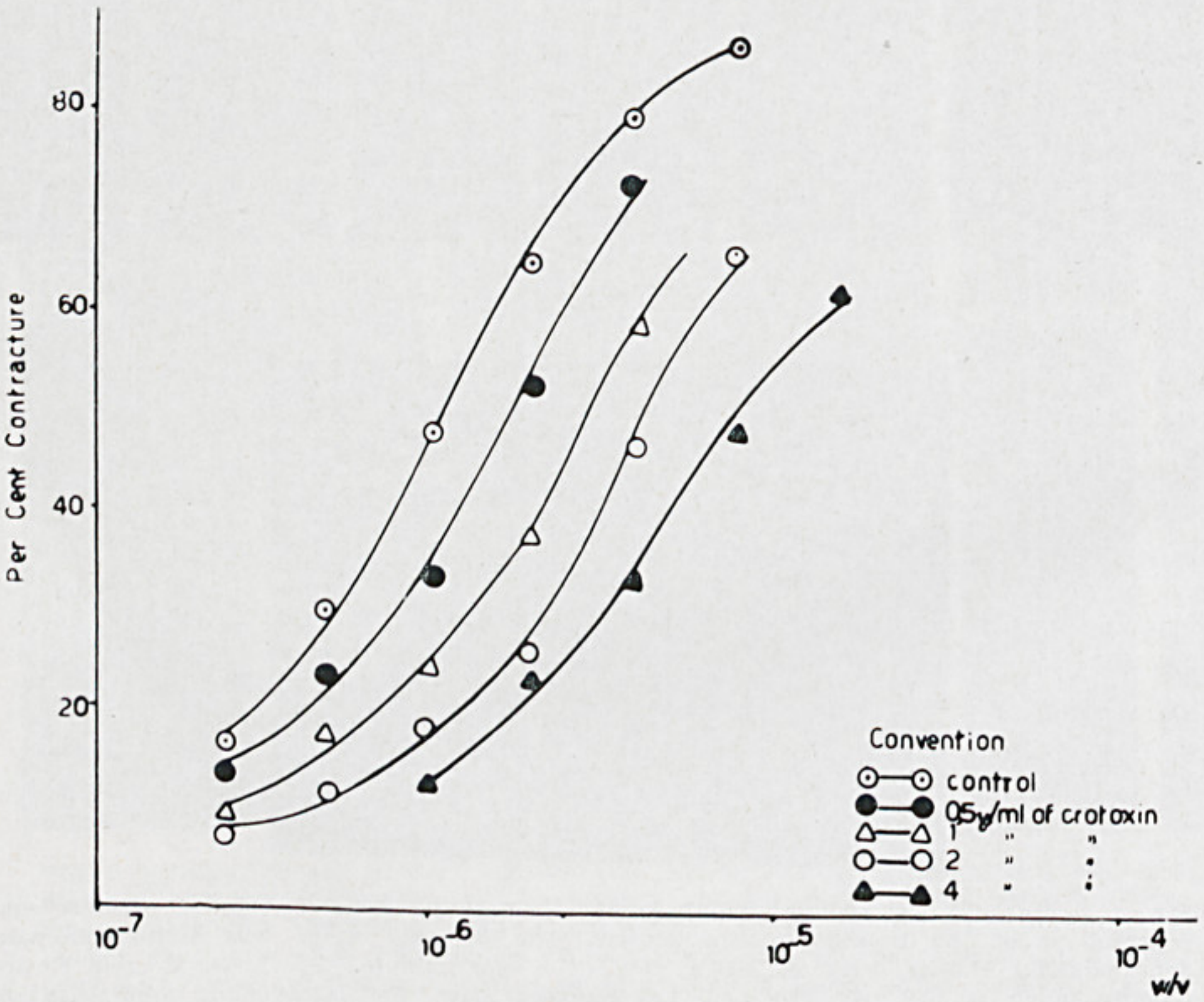


Fig. 5 — Dose-response curves for acetylcholine in the denervated rat hemi-diaphragm preparation in the absence of crotoxin and in the presence of various concentrations ($0.5 \times 10^{-6}$, $10^{-6}$, $2 \times 10^{-6}$ and $4 \times 10^{-6}$) of it. Each dose-response curve was obtained in a diferent denervated diaphragm as the effect evoked by crotoxin was, for pratical purposes, irreversible.

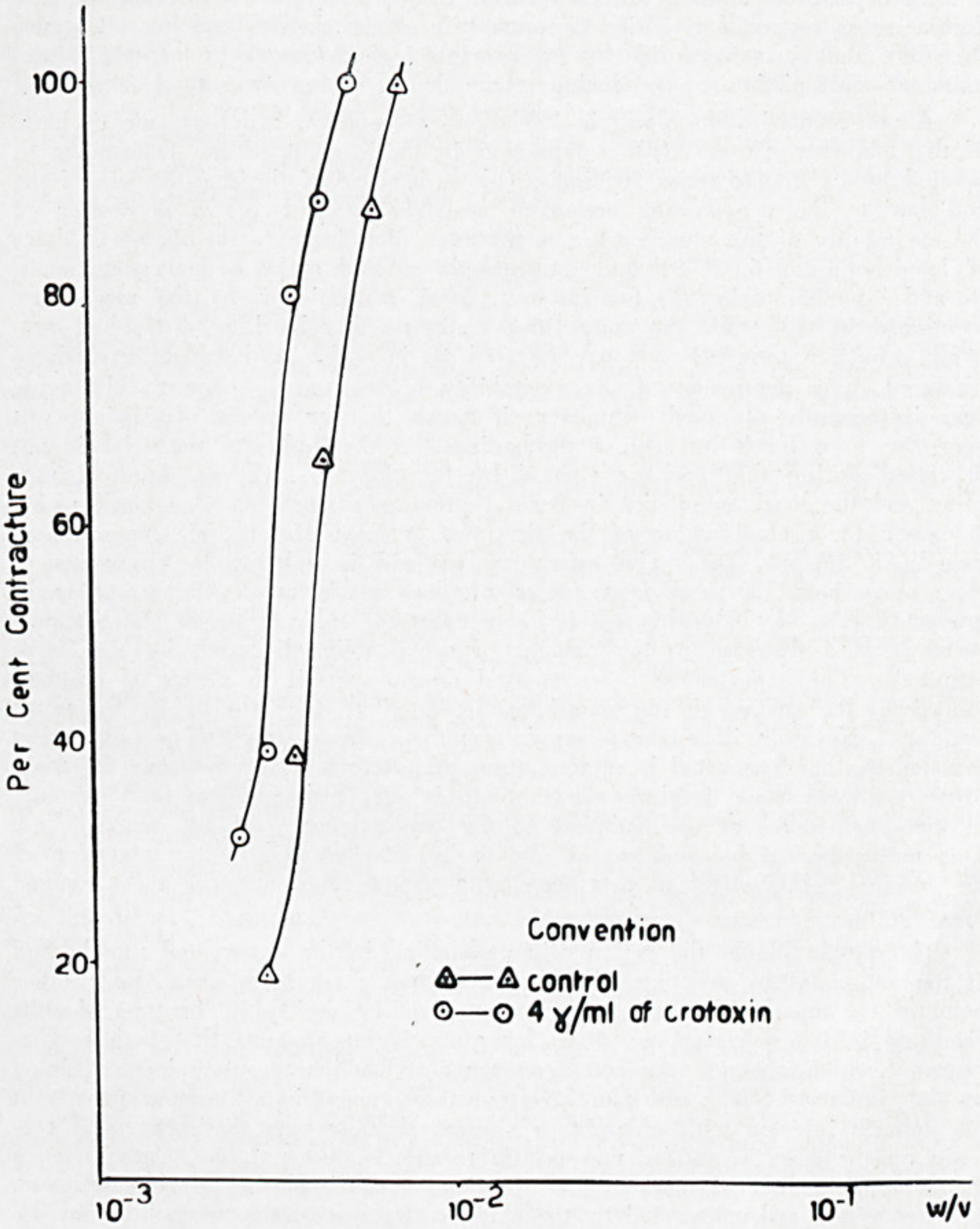


Fig. 6 — Dose-response curves for potassium chloride obtained in the denervated rat hemi-diaphragm in the absence of crotoxin and in the presence of $2 \times 10^{-6}$ of that substance. Crotoxin potentiated the action of the potassium ions on the denervated muscle.

these animals under crotoxin action were far from resembling those evoked by the depolarizing drugs. On the other hand, crotoxin in large doses produced a flaccid paralysis similar to that evoked by curare while large doses of the depolarizing drugs still give rise to contracture of the skeletal muscles. It seems, therefore, that it can confidently be concluded that crotoxin interrupts neuromuscular transmission by producing a non-depolarization type of blockade.

A non-depolarization block, in its largest acceptation, can have one or more of the following causes: (a) a reduction in the amount of the transmitter liberated by the motor nerve terminals, (b) a decrease of the sensitiveness of the end-plate to the depolarizing action of acetylcholine and (c) a depression of the excitability of the muscle fibre membrane. Botulinum toxin (5), small doses of hemicholinium (6, 7, 8) and the recently studied snake venom components, $\beta$- and $\gamma$-bungarotoxin (9), for instance, block transmission by the mechanism mentioned in (a) while the competitive neuromuscular blocking drugs and probably $\alpha$-bungarotoxin (9) by that referred to in (b). A blockade exclusively produced by a depression of the excitability of the muscle fibre membrane is very infrequently observed. However, it seems that an excess of calcium can give rise to a block by such a mechanism,, as the end-plate potencial is not depressed during the blockade elicited by this ion (10). Large doses of procaine and the local anesthetics in general provoke a neuromuscular block which is caused by a combination of the first two or even of all three factors mentioned (11, 12, 13, 14). The same can be said in relation to an excess of magnesium ions (15) and large doses of the antibiotics of the streptomycin group (16, 17, 18, 19), although in these cases the main cause of the blockade certainly is a decrease in the amount of acetylcholine liberated by the nerve terminals. The experiments here reported do not permit to decide if crotoxin diminishes the amount of the transmitter liberated at the end-plate by the nerve impulses. However, it can be inferred from the crotoxin-acetylcholine antagonism revealed in the denervated hemi-diaphragm preparation of the rat that the main cause of the blockade produced by crotoxin is very probably due to a decrease in the sensitiveness of the end-plate to the depolarizing action of acetylcholine. This mechanism of blockade seems also to be demonstrated by the reversion of the succinylcholine effect in cats showing a partial neuromuscular block evoked by crotoxin.

If crotoxin blocks the action of acetylcholine in the denervated muscle and at the end-plates by reacting with the acetylcholine receptors or at some other point of the muscle fibre membrane can not yet be decided. The type of shift produced in the dose-response curve for acetylcholine suggests that both mechanisms of action operate. An action on the acetylcholine receptors is also hinted by the similarity of the order of involvement of the various muscular groups in the animals injected with crotoxin or curare. In any case the fixation of crotoxin on the specific and/or non-specific receptors must not be a loose one as the combination of the majority of biologically active substances with receptors. This seems to be demonstrated by the extreme slowness of the reversibility of the paralysis and hence of the neuromuscular block produced by crotoxin.

Crotoxin exerts little or no effect on transmission at ganglionic synapses as well as at adrenergic neuroeffector junctions. This can be inferred from the results obtained in the experiments here reported concerning the contraction of the nictitating membrane in response to electrical stimulation of the sympathetic cervical chain in cats injected with large doses of crotoxin. As already stated, contractions of the nictitating membrane were always elicited in spite of the fact

that transmission at the myoneural junction was blocked one or more hours before the experiment. It is interesting to note that such selectivity of action on neuromuscular transmission seems to be only displayed by some curarizing drugs or some depolarizing agents. Gallamine and toxiferine, for instance, among the competitive neuromuscular blocking agents only paralyze ganglionic synapses when very large doses of them are administered to cats (20, 21). On the other hand, the agents which interrupts neuromuscular transmission by a pre-junctional mechanism such as botulinum toxin, hemicholinium, magnesium or streptomycin are usually quite as active in producing ganglionic blockade as they are in paralysing the myoneural junction.

## Summary

1. Similarity of the motor signs evoked by crotoxin and curare suggested the investigation of neuromuscular transmission in animals showing the paralysis elicited by crotoxin. The experiments were carried out on the sciatic-tibialis anterior muscle preparation of dogs to which crotoxin was injected with an antecipation of 24 or 48 hours. A partial or complete block of transmission was revealed in all experiments. Neostigmine, calcium, or bicarbonate were without effect on the blockade. However, the stimulation of the motor nerve with shocks of a high (50 c/s) frequency always caused a transitory post-tetanic improvement on transmission.

2. The neuromuscular blocking action of crotoxin was also studied in cats (sciatic-tibialis anterior muscle preparation). Large doses (0.5 mg/Kg and 1 mg/Kg) were employed. A complete or partial neuromuscular blockade appeared within the first three hours of crotoxin administration. Edrophonium and succinylcholine increased, although only to a small degree, the nearly completely blocked responses of the tibialis anterior muscle. Their effects were transitory.

3. Ganglionic transmission in crotoxin injected cats was investigated by stimulating the cervical sympathetic chain and recording the contractions of the nictitating membrane. The experiments were always done after transmission at the myoneural junction was blocked. The stimulation of the cervical sympathetic chain invariably elicited contractions of the nictitating membrane whose tensions did not decrease during the experiments. It seems, therefore, that no ganglionic blockade was caused by crotoxin.

4. The effect produced by crotoxin on the contractures evoked by acetylcholine or potassium in the isolated and cronically denervated rat hemi-diaphragm was investigated. Crotoxin shifted to the right the dose-response curve for acetylcholine. Only a slight decline of the slopes of the curves obtained in the presence of increasing concentrations of crotoxin ocurred. On the other hand, crotoxin did not antagonize, but potentiated, the action of potassium on the denervated muscle.

5. The following conclusions seem to emerge from the analysis of the results obtained on this investigation: I. Block of neuromuscular transmission is probably the sole cause of the paralysis evoked by crotoxin. II. Crotoxin interrupts neuromuscular transmission by producing a non-depolarization type of blockade. III. A decrease in the sensitiveness of the end-plate to the depolarizing action of acetylcholine is the unique or the main cause of crotoxin neuromuscular blockade. iv. Crotoxin probably reacts with the acetylcholine receptors and also at other sites of the muscle fibre membrane. Its fixation on the receptors can not be a loose one as the combination of the majority of biologically active substances with receptors.

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