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THE EFFECTS OF CURARE IN THE COCKROACH

I. dTC-INDUCED FAILURE OF LEG CONTRACTION

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INTRODUCTION

For many years insects were believed to be insensitive to curare (Harlow, 1958; Hopf, 1952; Krupp, Lendle & Stapenhorst, 1952; Roeder, 1948, 1953; Roeder & Weiant, 1950; Usherwood, 1963). However, Larsen, Miller & Yamamoto (1966) have demonstrated that intra-abdominal injection of d-tubocurarine chloride (dTC) into insects causes paralysis. The nature of this paralysis is of interest because the principles of both vertebrate and invertebrate physiology predict that curare should be without effect in insects. Since the work of Claude Bernard (1856), it has been generally maintained that curare acts specifically at the vertebrate, acetylcholine-mediated, neuromuscular junction to cause flaccid paralysis (Cutting, 1964). The neuromuscular junction of arthropods, in contrast, has been shown to be responsive to glutamic acid (Kerkut *et al.* 1965*a*; Kerkut, Shapira & Walker, 1965*b*), and, consequently, glutamic acid is reputed to be the transmitter at excitatory neuromuscular junctions in insects. As Cottrell & Laverack (1968) have stated in a recent review, the ability of curare to produce paralysis in insects means, 'that either ACh is the transmitter at the (neuromuscular) junctions, or that curare can block non-cholinergic synapses'.

Since the initial observation of curare-induced paralysis in insects by Larsen *et al.* (1966) two other reports have appeared concerned with the site and mechanism of curare action in insects. One of these reports (McCann, 1966) concludes that dTC acts at the neuromuscular junction of insects, while the other (Flattum, Friedman & Larsen, 1967) concludes that curare does not act at the neuromuscular junction but, rather, on some 'unidentified co-ordination centres'. The current investigation was therefore undertaken to determine the site of curare-induced paralysis in the cockroach, *Periplaneta americana*.

MATERIALS AND METHODS

A. Solutions administered

The solutions used in these experiments were: (1) Pringle's insect saline (Pringle, 1938), and (2) a curare solution consisting of 25 mg. dTC/ml. of Pringle's. dTC was obtained from Sigma Chemical Company (St. Louis, Missouri) in the crystalline state as d-tubocurarine chloride pentahydrate. Because of the insolubility of dTC in Pringle's saline, the curare solution was made by first dissolving dTC in de-ionized water, 50 mg./ml, and then mixing a known volume of this solution with an equal

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volume of twice-concentrated Pringle's. The solutions were stored under refrigeration and prepared anew every 10 days. Such precautions effectively precluded contamination. The solutions were brought to room temperature before being used in experiments.

B. Preparations Studied

Two different neuromuscular preparations were used in these studies, which permitted localized application of curare to different sites in the animal. The ventral preparation allowed the application and confinement of small volumes of solutions to the thorax. The dorsal preparation allowed the injection and confinement of a different solution into each of the metathoracic legs of one cockroach.

1. *Ventral preparation.* The ventral preparation is similar to that described by Kerkut, Shapira & Walker (1965*b*). A cockroach was pinned to a dissecting dish, ventral side uppermost, so that only its metathoracic legs were free to move. The legs were attached via thread to Harvard Apparatus Company Heart/Smooth Muscle modules (Harvard Apparatus Company), and leg contractions were recorded on a chart mover. The metathoracic ganglion was exposed and monophasic pulses from a Grass S-4 stimulator were delivered to the ganglion by lowering platinum-tipped, bipolar electrodes onto the ganglion with the aid of a micromanipulator. Such stimulation caused both legs to contract. The polarity of the electrodes was reversed periodically during test periods to prevent polarization of the electrodes.

2. *Dorsal preparation.* The dorsal surface of a cockroach was bonded to a glass rod with all but the metathoracic legs immobilized. The glass rod with cockroach attached was then clamped to the undersurface of a small table so that the animal's dorsal surface was uppermost. The tarsal segments of the metathoracic legs were removed and cotton thread was secured around the tibiae. The legs were attached via thread to Heart/Smooth Muscle modules, and contractions were recorded on a chart mover. To permit injection of solutions into each of the metathoracic legs, fine cannula tubing (Clay Adams Intramedic PE/50 tubing) was secured in the coxa of each leg. Measured volumes of solutions could then be delivered to a leg by placing the needle of a microlitre syringe into the free end of the cannula tubing and delivering the desired volume.

The metathoracic legs of dorsal preparations were caused to contract either by stimulating the metathoracic ganglion or by stimulating the leg. In the latter case the metathoracic ganglion was removed and current was delivered across the coxal-femoral joint of each leg. Silver wire 0·002 mm. in diameter was wrapped around the coxa and femur of each leg, and a conducting solution* was used to enhance electrical contact between wire and leg. The leg which responded to stimulation with the greater amplitude of leg contraction was injected with curare solution, while the opposite leg was injected with Pringle's saline.

RESULTS

A. Contraction failure caused by intra-abdominal injection of curare

The effect of intra-abdominally injected curare was studied in both the ventral and the dorsal preparations. As shown in Figure 1 A, the ventral preparation responds to ganglionic stimulation with uniform contraction of both metathoracic legs for long

* The formulation of conducting solution appears in Friedman (1969).

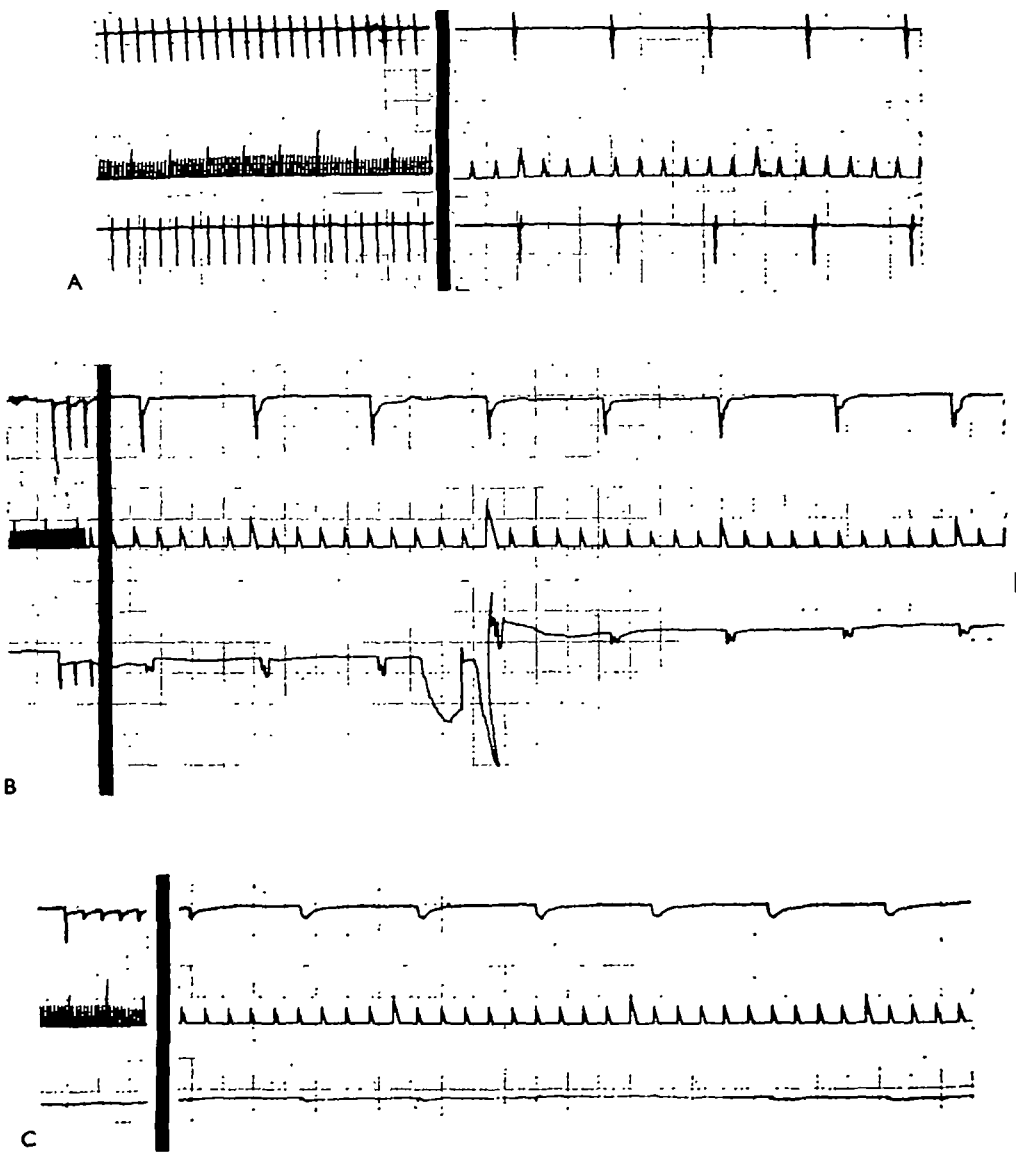

Fig. 1. The effect of intra-abdominal injection of curare on leg contraction in response to ganglionic stimulation in three different cockroaches. In this and subsequent figures the recordings are read from left to right. The responses of the metathoracic legs are indicated by the upper and lower traces of each recording. Upward deflexions from the centre trace indicate time marked in seconds. A. Normal contraction in response to ganglionic stimulation recorded 330 min. after preparation isolated. (Parameters of stimulation: f = 0·25 pulses/sec., d = 25 msec., V = 2·5 V. Chart speeds 0·7 mm./sec., and 3·8 mm./sec.) B. Response to ganglionic stimulation 60 min. after an intra-abdominal injection of 1·1 μg dTC/mg. body wt. (Parameters of stimulation: f = 0·2 pulses/sec, d = 25 msec., V = 2·5 V. Chart speeds same as in A). C. Response to ganglionic stimulation 285 min. after an intra-abdominal injection of 1·1 μg. dTC/mg. body wt. (Parameters of stimulation and chart speeds same as in B.)

periods of time. Intra-abdominal injection of dTC impairs this response by increasing the amount of time the leg remains flexed and by causing a progressive diminution in the height of contraction. This can be seen by comparing Fig. 1 B with

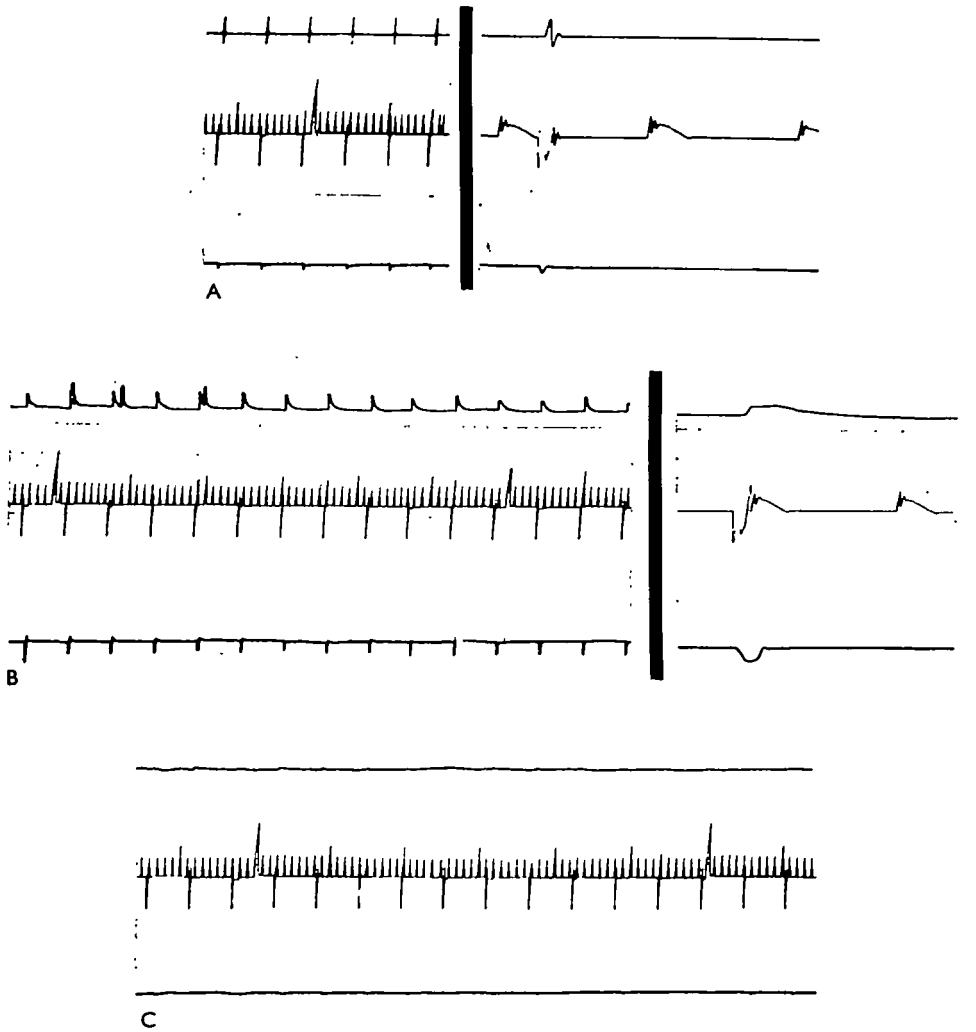

Fig. 2. The effect of intra-abdominal injection of curare on leg contraction in response to stimulation across the coxal-femoral joint of a cockroach. The metathoracic ganglion of the preparation has been removed. In this and subsequent figures downward deflexions from the centre trace mark the events of stimulation. Except where noted, chart speeds are: 1·2 mm./sec. and 24 mm./sec. (Parameters of stimulation: $f = 0{\cdot}2$ pulses/sec, $d = 3$ msec., $V = 50$ V.) A. Normal response to direct stimulation across the coxal-femoral joint recorded 110 min. after preparation isolated. B. Response to direct stimulation 95 min. after intra-abdominal injection of 25 μl. of curare solution. C. Response to direct stimulation 245 min. after intra-abdominal injection of 25 μl. of curare solution.

Fig. 1 C. Eventually, contraction fails completely. Intra-abdominal injection of an equivalent amount of Pringle's saline caused no alteration in contraction (not shown).

Figure 1 also demonstrates that the impairment of contraction seen after dTC

injection into a ventral preparation is not caused by fatigue of the preparation. (1) Fig. 1 A shows that ventral preparations are capable of contraction for extended periods after isolation, (2) Figs. 1 B and 1 C are the responses of newly isolated, and therefore non-fatigued, preparations.

The metathoracic ganglion need not be present for curare-induced failure of contraction to occur. This is shown in Fig. 2. When metathoracic legs are deprived of their innervating ganglion and stimulated directly across the coxal-femoral joint, they respond with uniform contractions lasting for long periods of time (Fig. 2 A). Comparison of Fig. 2 A with Fig. 1 A reveals the similarity of contractions to ganglionic and to direct stimulation. Figs. 2 B and 2 C demonstrate that the injection of curare impairs the contraction of legs directly stimulated. The impairment seen is similar to that observed when the metathoracic ganglion is present and stimulated. (1) The leg remains flexed for an increased amount of time, (2) the height of the contraction is diminished and (3) eventually contraction fails completely.

B. Contraction failure caused by injection of curare into a leg

Localized injection of curare caused failure of contraction in a metathoracic leg. The contraction failure is similar to that caused by intra-abdominal injection. Fig. 3 monitors the contraction of both metathoracic legs of a dorsal preparation to ganglionic stimulation before and after the administration of curare to one of these legs. Fig. 3 A shows the initial contraction before injection. The injection of 25 μl. of curare solution into the leg of the upper trace, (1) increased the time for which the leg remained flexed (Fig. 3 B), (2) decreased the height of contraction (Fig. 3 B) and (3) eventually caused failure of contraction (Fig. 3 C). The simultaneous injection of 25 μl. of Pringle's saline into the contralateral leg (lower trace) was without effect.

The contraction failure observed in a curare-injected leg was reversible. As shown in Fig. 3, repeated saline injections into a curare-blocked leg were able to restore contraction in response to ganglionic stimulation (Fig. 3 D).

C. Contraction failure caused by application of curare to the metathoracic ganglion

Reversible failure of contraction in the metathoracic legs was also achieved by applying 25 μl. of curare solution to the metathoracic ganglion of a ventral preparation. This is shown in Fig. 4. By virtue of the elevated position of the metathoracic legs of this preparation, and the small volume of curare solution applied, it is unlikely the dTC diffused into the legs and acted peripherally. This interpretation is further supported by the observation that 25 μl. of dye solution, when applied to the metathoracic ganglion, does not find its way into the metathoracic legs.

Fig. 4 A shows normal contraction in response to ganglionic stimulation 35 min. after the application of saline to the metathoracic ganglion. The application of 25 μl. of curare solution to the metathoracic ganglion, (1) increased the amount of time the legs remained flexed, (2) decreased the heights of contraction and (3) caused contraction failure. This is shown in Fig. 4 B. Contraction failure was reversed by periodically irrigating the metathoracic ganglion with saline. As shown in Fig. 4 C, recovery was seen 75 min. after saline irrigation was initiated.

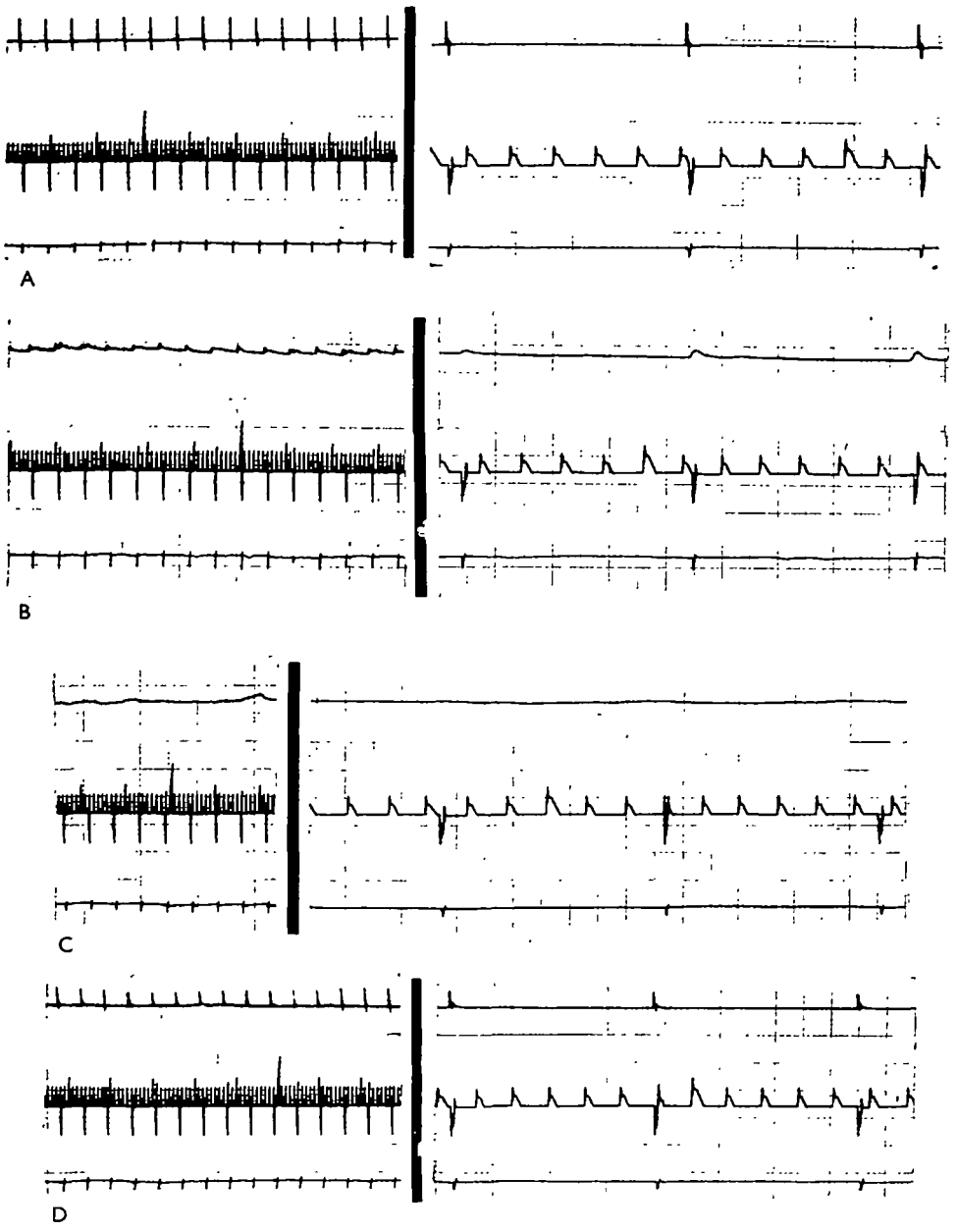

Fig. 3. Injection of curare into a leg of a dorsal preparation causes reversible failure of contraction. (Parameters of stimulation: f = 0·2 pulses/sec., d = 25 msec., and V = 2·5 V. Chart speeds 0·7 mm./sec. and 7·0 mm./sec.). A. Normal contraction in response to ganglionic stimulation recorded 20 min. before 25 μl. of curare solution (test solution) was injected into leg of upper trace and 25 μl. of Pringle's saline (control solution) was injected into leg of lower trace. B. Response to stimulation 10 min. after legs were injected with test and control solutions. C. Response to stimulation 105 min. after legs were injected with test and control solutions. D. Response to stimulation 105 min. after initiation of repeated injections of saline into both metathoracic legs.

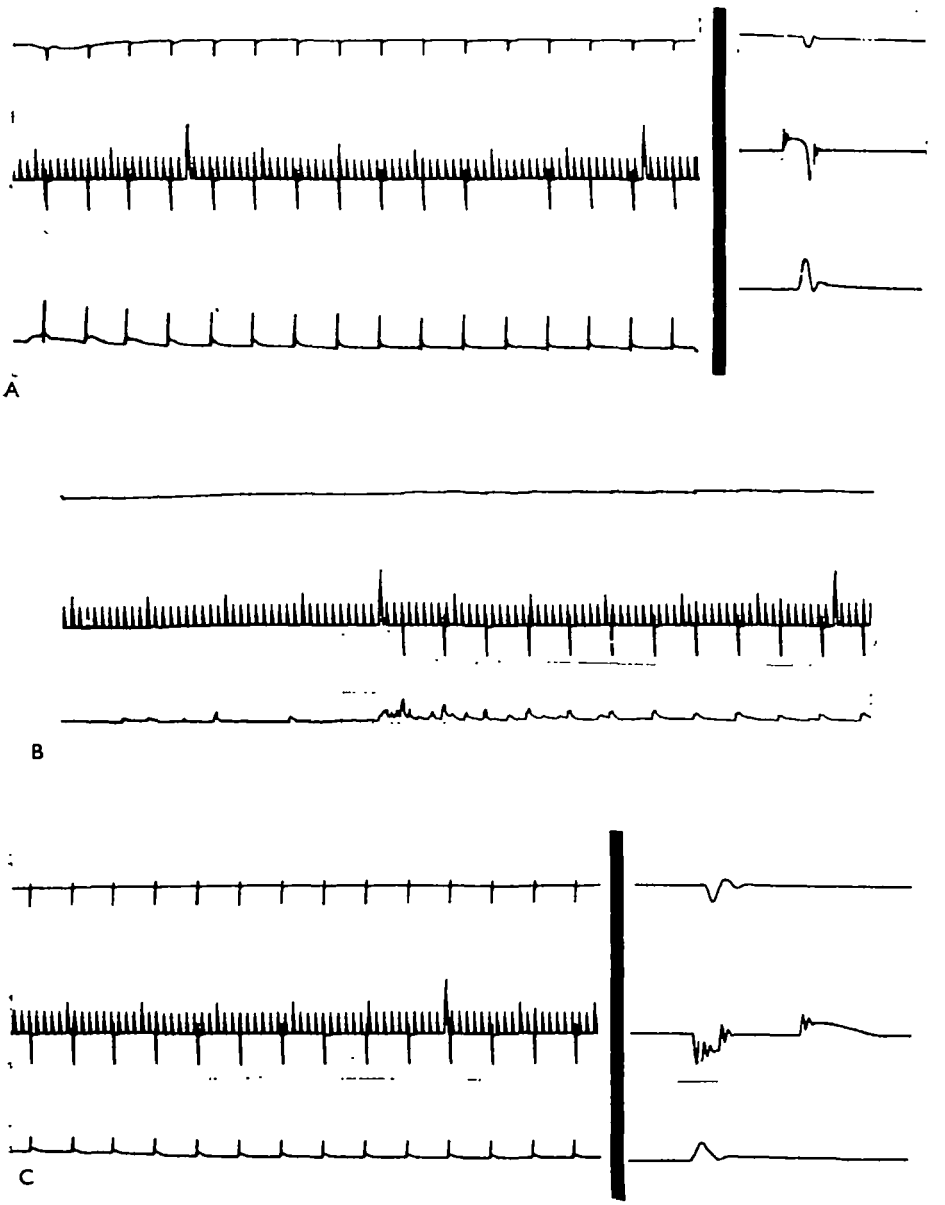

Fig. 4. Application of curare to the metathoracic ganglion causes reversible failure of contraction. (Parameters of stimulation: $f = 0{\cdot}2$ pulses/sec., $d = 25$ msec., $V = 2{\cdot}5$ V.) A. Normal contraction in response to ganglionic stimulation 35 min. after saline applied to the metathoracic ganglion. B. Response to stimulation 15 min. after 25 μl. of curare solution was applied to the metathoracic ganglion. C. Response to stimulation 75 min. after initiation of periodic irrigation of the metathoracic ganglion with saline.

DISCUSSION

The contraction failure observed after the injection of curare into a cockroach is, for the following reasons, most probably due to a peripheral blocking action of curare. (1) The metathoracic ganglion need not be present for contraction failure in the metathoracic leg to result from intra-abdominal injection of curare, (2) local application of curare to the leg produces contraction failure similar to that caused by intra-abdominal injection and (3) the curare effect is reversible (Figs. 3 and 4) and therefore is not due to fatigue of the preparation, but rather is due to a direct effect of the drug.

The results of the current investigation, therefore, argue against previous hypotheses concerned with the mechanism of insect curarization. There is little likelihood that the curare-induced failure of contraction in the cockroach originates within the central nervous system or some other co-ordination centre as Flattum *et al.* (1967) have proposed for the cricket, *Acheta*. It seems equally unlikely that curare must form a complex in the cockroach (with a component of the circulatory system, or with a substance from the tissues of the abdomen) to be active. Such a proposal was made by McCann (1966) based upon her finding that injected doses, but not directly applied doses of curare, were effective in blocking the muscle action potential of the flight muscle of *Sarcophaga*.

How then does curare manifest its peripheral action? Roeder & Weiant (1950) have demonstrated that the leg musculature of *P. americana* can not be directly excited by electrical stimulation. Rather, electrical stimuli delivered to a cockroach leg depolarize the motor nerves within the leg, which, in turn, leads to the release of neuromuscular transmitter and the subsequent contraction. Thus, the ability of curare to reversibly block insect leg contraction is most probably due to any of three possible mechanisms of curare action. (1) dTC protects the post-synaptic transmitter receptor, (2) dTC prevents the pre-synaptic transmitter from being released and (3) dTC prevents impulses in the motor axon from invading the neuromuscular junction, thereby preventing the release of transmitter.

Despite several investigations into the effect (or lack of effect) of dTC on the insect neuromuscular junction, there is no evidence indicating that curare either protects the post-synaptic receptor or prevents transmitter release. McCann (1966) could not alter the muscle action potentials of the fly *Sarcophaga* by the direct application of dTC to the muscle (and endplate). Usherwood (1963) could not alter the mEPP's of flexor and extensor tibiae muscles of *P. americana* with 10^{-2} M dTC. Roeder & Weiant (1950) found that 10^{-3} M dTC failed to alter the contraction of the tergal leg muscles of *P. americana*. The inability of curare to affect the neuromuscular junction is further supported by the evidence (Kerkut *et al.* 1965*a*, *b*) that glutamic acid, not acetylcholine, is the transmitter of the excitable neuromuscular junctions of insects.

The remaining possible explanation for curare-induced failure of contraction is therefore the following: dTC prevents impulses in the motor nerve from invading the neuromuscular junction. Support for this hypothesis comes from the present finding that curare-induced failure of contraction exhibits the same characteristics regardless of whether the dTC is applied to the metathoracic ganglion or to the metathoracic leg. Structures which should be suspected as being responsible for the contraction failure are those involved in the contraction response, and are present in both the peripheral

region of the leg and in the region of the metathoracic ganglion. The only structures which meet these qualifications are the motor nerves. It seems reasonable to postulate that contraction failure is caused by conduction block in the motor axons. The conduction block may occur as the motor nerves leave from the metathoracic ganglion or in the peripheral region of the leg.

The ability of curare to produce conduction block in nerve axons has been previously demonstrated in the giant axon of the squid (Rosenberg, 1965; Rosenberg & Ehrenpreis, 1961 *a*, *b*; Rosenberg & Hoskin, 1965; Rosenberg & Podleski, 1962, 1963), in frog nerve fibres (Dettbarn, 1960; Walsh & Deal, 1959) and in the leg nerve of the lobster (Dettbarn, 1963). There are no previous reports of curare-induced conduction block of insect nerve fibres. However, the results of both McCann (1966) and Suga & Katsuki (1961) are suggestive of curare-induced blockage of nerve impulses. McCann (1966) was unable to demonstrate an effect when curare was applied directly to muscle, but was able to demonstrate an effect when curare was injected into the insect. These results are compatible with the hypothesis that curare does not act on insect muscles or their endplates, but rather on the nerve fibres. Suga & Katsuki (1961) were able to demonstrate that perfusion of the prothoracic ganglion of a grasshopper with dTC blocked auditory impulses in the central nervous system. These results suggest that curare may be capable of blocking impulses in the tympanic nerve as it enters the prothoracic ganglion. The ability of curare to block impulses in nerve fibres of the cockroach will be demonstrated in a subsequent report.

SUMMARY

1. The nature of insect curarization has been investigated in the cockroach, *P. americana*. Mechanical studies of leg contraction revealed that dTC, whether injected into the abdomen, injected into a leg or applied to the metathoracic ganglion, produces failure of contraction.

2. The contraction failure caused by injecting dTC into a leg or by applying dTC to the metathoracic ganglion could be reversed by washing the drug out of the affected area.

3. The central nervous system does not appear to be essential for curare-induced contraction failure. The contraction of metathoracic legs deprived of their metathoracic ganglion is abolished in the presence of curare.

4. Since curare produces contraction failure when applied to the metathorax and when injected into a leg, the site of curare action must be present in both these locations. The motor nerve fibres are present in both these locations and it is proposed that contraction failure is due to the action of curare on these fibres.

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