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ACTIONS OF SCORPION VENOM ON SKELETAL MUSCLE

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A protein-like constituent of scorpion venom produced effects on the skeletal muscle fibre resembling those of citrate, lack of calcium or veratrine. These effects could be diminished by the addition of calcium. They do not appear to be due to the formation of a non-ionized complex with calcium.

A notable feature of the experimental poisoning of laboratory animals with scorpion venom is the occurrence of irregular, frequent twitches and tetanic contractions of skeletal muscle. Wilson (1904 1921; cited by Rohayem, 1953) found that venom had a direct stimulating action on skeletal muscle, and that it caused a block of neuromuscular transmission which he considered to be unlike the block produced by curare.

The experimental work described in this paper was undertaken to investigate these findings more fully.

METHODS

The methods of obtaining, storing, and redissolving the venom have been described previously (Adam and Weiss, 1958). In all the experiments described the venom used was that of *Leiurus quinquestriatus* and all doses quoted refer to dried, whole venom from a pooled sample. The venoms of *Buthotus minax* and *Parabuthus hunteri* were also investigated and found to have similar action on the isolated muscle preparations.

Isolated Toad Sartorius Muscle Preparation.—The sartorius muscle of *Bufo regularis* was dissected out with the motor nerve cut at the entrance into the muscle, and put into a 1 ml. bath containing Ringer solution at room temperature (25°±4°). The composition of the Ringer solution (mM.) was: NaCl 116, KCl 2.0, $CaCl_2$ 1.8, $NaHCO_3$ 3.0. During the experiments the whole content of the bath was replaced by the relevant test solutions. When it was necessary to alter the concentration of calcium, the isotonicity was maintained by changing the concentration of NaCl. The contractions were recorded on a smoked paper using a spring-loaded lever with an amplitude magnification of 10.

Isolated Rat Diaphragm Preparation.—The method was that of Bülbring (1946), except that Krebs solution of the following composition was used (mM.): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, dextrose 11. The muscle was suspended in a 60 ml. bath at 37°. The *p*H remained between 7.2 and 7.3 when a mixture of 95% O_2 and 5% CO_2 was bubbled through the bath. In the experiments with an altered calcium concentration, isotonicity was maintained by changing the concentration of NaCl. Electrical stimulation was effected by an electronic stimulator delivering rectangular pulses through platinum electrodes at the rate of 0.2/sec. The preparation was stimulated either through the phrenic nerve or directly, in which case tubocurarine (0.2 μg./ml.) was added to the bath.

Isolated Toad Cardiac Muscle Preparation.—A strip of cardiac muscle approximately 1.5×1.5×15 mm. was cut from the ventricle and suspended in Ringer solution of the same composition as that used for the sartorius muscle, in a 1 ml. bath at room temperature (25°±4°). The strip was electrically stimulated by rectangular pulses at 0.2/sec. through two platinum electrodes.

Electrophoresis of Venom.—Electrophoresis of venom was carried out on Whatman No. 31 paper in 0.25 N-acetic acid, *p*H 2.6, at 0.25 mA./cm., or in borate buffer, *p*H 8.6, at 0.6 mA./cm. The paper was then cut longitudinally and one half of it treated with 0.2% (w/v) ninhydrin in acetone containing 10% (w/v) acetic acid (Jepson and Stevens, 1953), and subsequently heated at 90°. When the paper was viewed in ultra-violet light, 5-hydroxytryptamine as well as constituents giving the usual ninhydrin reaction (Adam and Weiss, 1958) were demonstrated. The other half of the strip was cut into the fractions indicated by the bands on the stained half, and these were eluted overnight in Ringer or Krebs solution at 4°. Other dyeing procedures used were the azocarmine method (Turba and Enenkel, 1950) and the amidoschwarz method (Grassmann and Hannig, 1950). Eluates were obtained as above.

RESULTS

Isolated Toad Sartorius Muscle.—The immersion of a sartorius muscle in a solution of venom (10 μg./ml.) in Ringer produced after a delay of 2 to 3 sec., a moderately rapid contracture upon which, at the peak of the amplitude, frequent short irregular twitches were often superimposed. The

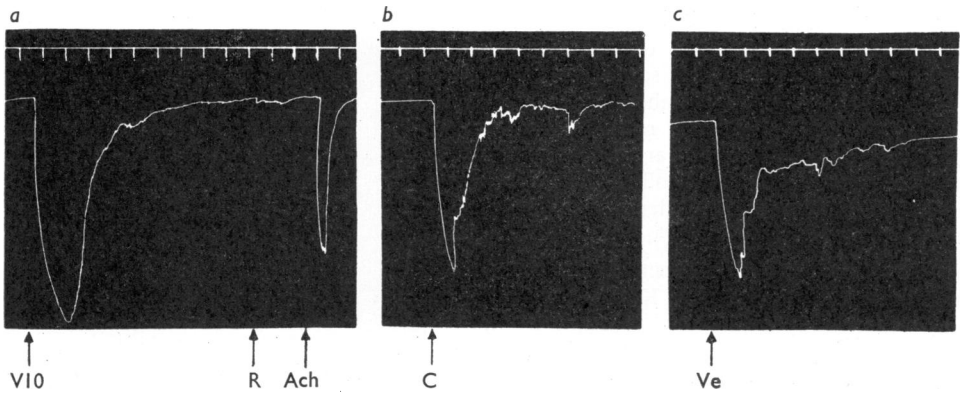

FIG. 1.—Comparison of effects of venom, veratrine, and citrate on the isolated toad sartorius muscle, isotonic recording. *a*: venom 10 μg./ml. (V10). R, refilled with fresh venom at same concentration. Ach, 2 μg./ml. of acetylcholine. *b*: C, sodium citrate 2.5 mM. *c*: Veratrine 0.2 mg./ml. (Ve). Time, 30 sec.

increased tone and the twitching continued for about 25 to 35 sec. Subsequently the tone fell slowly and irregularly, and the amplitude and frequency of the twitches diminished. Within 3 to 5 min. after the initial exposure to the venom, the muscle, still in the venom solution, had usually regained its original tone, but small, irregular twitches could be seen for periods up to 20 min. Draining of the bath and immediate refilling with a fresh solution of venom in the previous concentration did not produce a contracture, but other stimuli, such as acetylcholine or electrical stimulation, retained their effectiveness (Fig. 1*a*). Muscles which had been in contact with solutions of venom for more than about 30 sec. did not completely regain their original responsiveness to venom even if they were washed 3 to 4 times and allowed to remain in normal Ringer solution for as long as 30 min. Repeated exposure to venom at short intervals (1 min.), with two intermediate washings in Ringer, resulted in rapid tachyphylaxis; but after less than 30 sec. contact with venom, muscles regained their original responsiveness almost completely when washed and allowed to remain in normal Ringer for longer than 7 to 8 min. (Fig. 2).

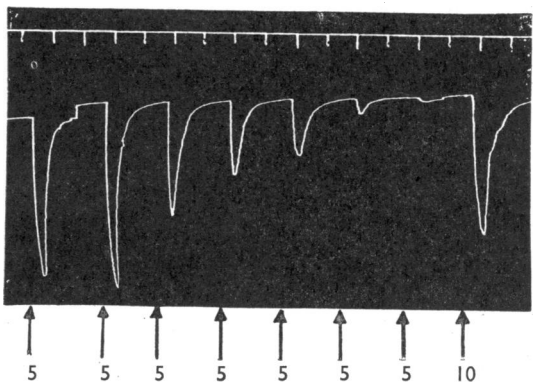

FIG. 2.—Isolated toad sartorius muscle. 5: Venom 5 μg./ml., 10: Venom 10 μg./ml., each in contact with muscle for 15 sec. Between first two doses, 8 min. in Ringer solution free of venom. Intervals of 1 min. between subsequent doses. Time, 30 sec.

The exposure of muscles to higher concentrations of venom produced more rapid contractures with shorter delay periods and greater amplitudes. At concentrations above about 16 μg./ml. of venom maximal twitches were often superimposed on the contracture: lower concentrations of venom produced smaller contractures after a longer delay (Fig. 3). Venom concentrations

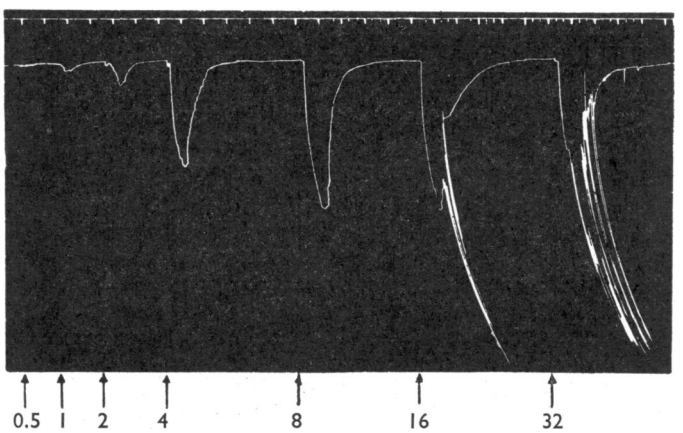

FIG. 3.—Isolated toad sartorius muscle. Effects of 0.5, 1, 2, 4, 8, 16, and 32 μg./ml. of venom, each in contact with muscle for 15 sec. The numerals indicate the amounts of venom. Time, 30 sec.

below 2.0 μg./ml. did not produce consistent responses, but concentrations of 0.5 or 1.0 μg. venom/ml. sometimes produced small irregular twitches, without any increase in the muscle tone after a delay of 30 sec. to several minutes. After the addition of sufficient tubocurarine to repress completely the response to acetylcholine (2 μg./ml.), the muscles did not show any alteration in their responses to venom.

Isolated Rat Diaphragm.—The effects of immersing the isolated rat diaphragm in Krebs solution containing venom did not differ in principle from those on the toad sartorius muscle, but the contracture was better maintained (Fig. 4). Smaller

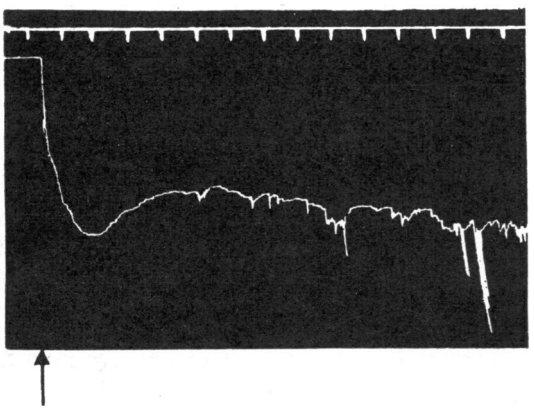

FIG. 4.—Rat diaphragm. At arrow, 10 μg./ml. of venom. Time, 30 sec.

concentrations of venom, as low as 1.0 μg./ml., caused reproducible contractures. After repeated contact at short intervals, tachyphylaxis to venom appeared in the same way as with the sartorius muscle. The effects of venom could be reversed by washing if the time of exposure to the venom was kept short, not exceeding 1 min. With higher concentrations of venom (10, 15, and 20 μg./ml.), the responses had shorter delays and greater amplitudes. After the addition of tubocurarine (2 μg./ml.) to the bath, the response to venom was unaltered.

Modification by Venom of the Effects of Electrical Stimulation of Rat Diaphragm.—When venom was applied to the bath during direct electrical stimulation of the preparation at 5/min., the response was modified as follows: (1) The amplitude of the electrically-induced contractions was increased at first, and later diminished (Fig. 5*a*). This increase in amplitude was superimposed on the contracture previously described. It was seen only when the electrical stimuli applied were submaximal, suggest-

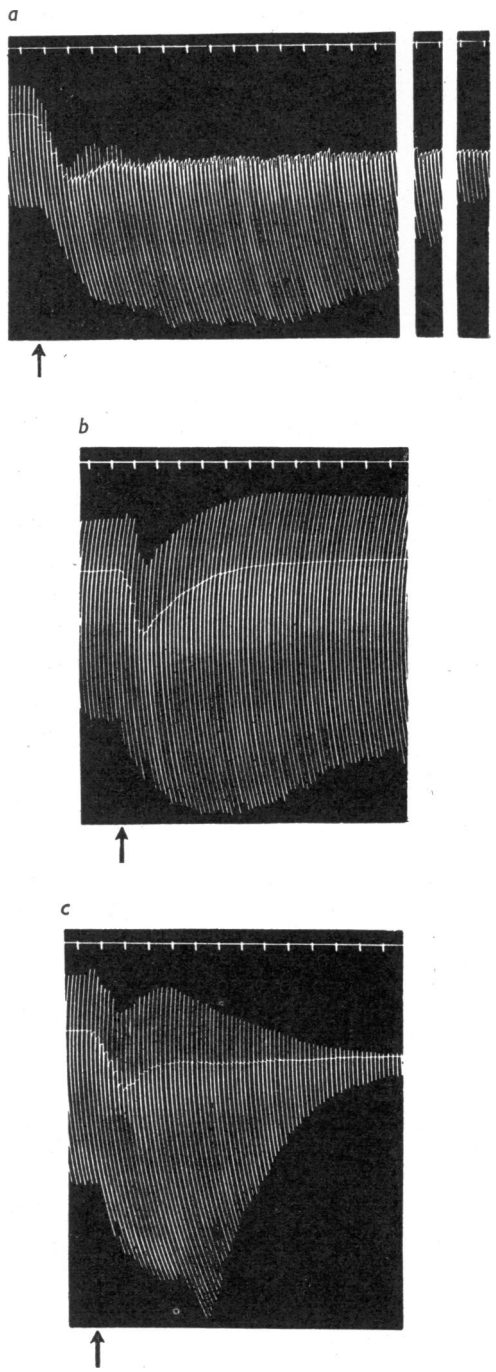

FIG. 5.—Rat diaphragm. Direct submaximal stimulation. *a*: 5 μg./ml. of venom at arrow. Between the last two records 15 min. elapsed. *b*: 10 mM. sodium citrate at arrow. *c*: 35 μg./ml. of veratrine at arrow. Time, 30 sec.

ing that the effect was due to a lowering of the threshold rather than to an increased contractility. (2) The rate of relaxation was decreased. This appeared to be connected with small spontaneous twitches induced by the venom which were very obvious when watching the irregular return of the writing lever after each induced contraction. A slower rate of relaxation was also indicated by a disappearance of the rebound overshoot of the recording lever.

Similar effects were produced by venom when the rat diaphragm was stimulated indirectly through its phrenic nerve.

Comparison of the Effects of Venom, Citrate, and Veratrine.—The effects of venom on the striated muscle preparations bore some resemblance to those of calcium-complexing agents and to those of veratrine. We compared therefore the effects of venom, sodium citrate, and veratrine on the toad sartorius muscle and the rat diaphragm.

On the toad sartorius muscle, the three agents produced a qualitatively similar effect, consisting of a contracture followed by a slow irregular relaxation, accompanied by twitching, whilst the agents remained in the bath (Figs. 1*a*, *b*, *c*). On the electrically-stimulated rat diaphragm the three agents gave a contracture and an initial increase in amplitude of the twitches, followed by a decrease, but the time relation of this effect differed. When doses of the three agents which induced approximately equal contractures were used, the subsequent depression set in most rapidly with veratrine and least rapidly with citrate, the effect of venom being intermediate. A further difference was that the slowing of relaxation and the twitching, which are characteristic features of the action of venom, were absent when veratrine or citrate was used (Figs. 5*a*, *b*, *c*).

When veratrine was applied during the depressant phase of venom action, the muscles were found to be more sensitive, and a contracture could be produced with only about a quarter of the veratrine dose normally required to produce the same effect on a fresh muscle. Muscles did not become more sensitive to venom during contact with veratrine.

Effects of Changes in Calcium Concentration.—Since citrate lowers the level of ionized calcium, and since displacement of calcium from binding sites at excitable membranes has been suggested as an explanation of the action of veratrine (Gordon and Welsh, 1948), the effects of changes in the calcium concentration in the bath fluid on venom action were investigated.

On both the sartorius muscle and diaphragm, high concentrations of calcium diminished the effects of venom. Doubling the calcium concentration diminished the venom effect on the sartorius muscle, and this diminution increased with time (Fig. 6). A four-fold increase in the calcium concentration completely abolished the effect of 10 μg./ml. of venom. On the rat diaphragm, venom antagonized the depression by high calcium concentrations of the electrically-induced contractions. Fig. 7 shows the depression produced by a four-fold increase in the calcium concentrations, and the

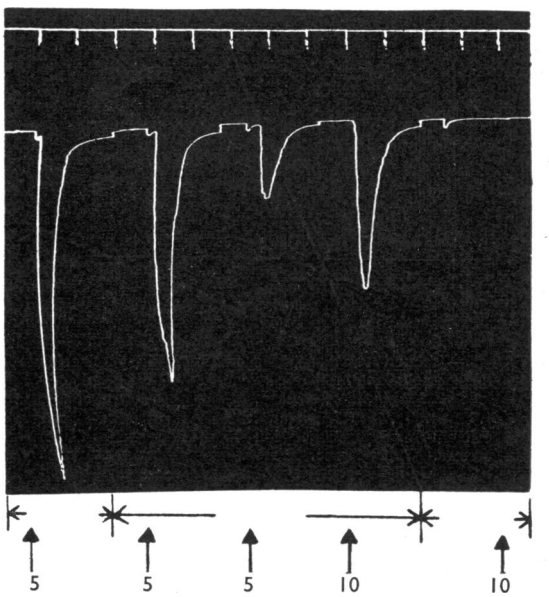

FIG. 6.—Isolated toad sartorius muscle. Effect of venom in presence of increasing calcium concentration. 10 min. elapsed between each dose of venom. **5**: 5 μg./ml. of venom. **10**: 10 μg./ml. of venom. The calcium concentration was doubled at each pair of horizontal arrows. Original concentration 1.8 mM. Time, 30 sec.

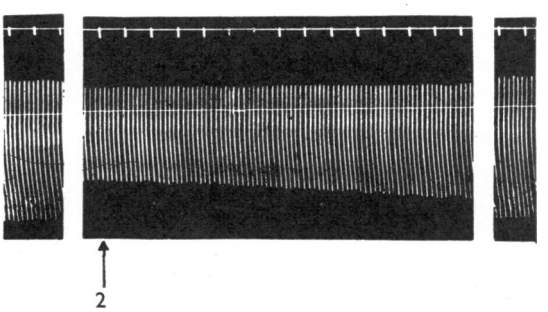

FIG. 7.—Rat diaphragm. Direct submaximal stimulation. Effect of 1.8 mM. calcium in the first record. In the second and third records, the calcium concentration was increased four-fold. Between the first and second record 1 min. elapsed, and between the second and third 6 min. elapsed. **2**: 2 μg./ml. of venom. Time, 30 sec.

restoration by venom of the amplitude of the contractions.

In Ringer solution containing no calcium, the sensitivity of the toad sartorius muscle to venom was increased (Fig. 8): the sensitivity of the electrically-stimulated rat diaphragm to venom increased so that 0.5 μg./ml. of venom caused a distinct contracture. Further, if a diaphragm had been in contact with a concentration of venom not normally producing a contracture (say, 0.5 μg./ml.), the subsequent immersion of the preparation in Krebs solution containing neither calcium nor venom gave a contracture (Fig. 9).

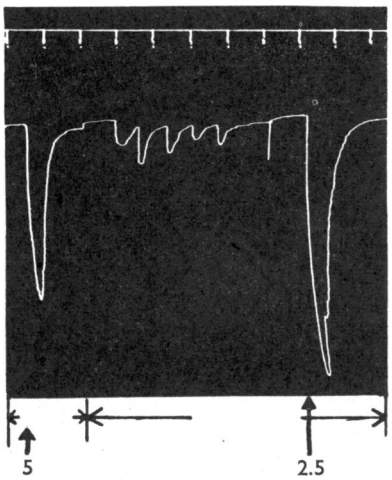

FIG. 8.—Isolated toad sartorius muscle. Effect of calcium-free Ringer solution on that of venom. **5**: 5 μg./ml. of venom. **2.5**: 2.5 μg./ml. of venom. Original calcium concentration 1.8 mM. between left-hand horizontal arrows. Changed to calcium-free Ringer between right-hand pair of horizontal arrows. Time, 30 sec.

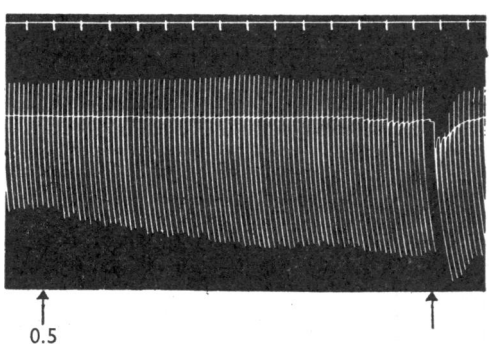

FIG. 9.—Rat diaphragm. Direct submaximal stimulation. Effect of immersion in calcium-free Krebs at second arrow after previous contact with a small dose of venom at first arrow. **0.5**: 0.5 μg./ml. of venom in Ringer solution of normal composition. Time, 30 sec.

When toad cardiac muscle was placed in calcium-free Ringer solution, the threshold to electrical stimulation was promptly lowered and, within a short time, the amplitude of the contractions decreased. Restoration of the normal calcium-concentration gave a rapid reversal. The addition of citrate 5 mM./l. to Ringer solution had a similar effect on the threshold and on the amplitude of the contractions as the absence of calcium from the bath fluid and was also fully reversible. Venom in concentrations of 5 to 60 μg./ml. markedly lowered the threshold to electrical stimulation, but neither affected the amplitude of the contractions nor produced a contracture.

Nature of the Active Principle in Venom.—When kept in a desiccator for several months the crude, dried venom did not lose any activity as measured by contractions which it produced in the sartorius muscle and rat diaphragm preparations. In aqueous solution (1 mg./ml.) at 4°, activity was maintained for 1 to 2 weeks. Venom solutions (20 μg./ml.), heated in a boiling water bath at *p*H 7 for 10 min., lost 80% of activity on sartorius muscle and rat diaphragm. Complete loss of activity occurred after heating for 30 min. in a boiling water bath at *p*H 7. Incubation of venom (10 μg./ml.) with trypsin or chymotrypsin (100 μg./ml.) for 30 min. at 37° at *p*H 7.4 resulted in a complete loss of activity on the muscle preparations. The active constituent did not dialyse detectably through cellophane in 24 or 48 hr. at 4° against 5 volumes of Ringer solution or distilled water changed 4 times. The crude venom could be split by electrophoresis into several fractions giving a violet reaction with ninhydrin. With azocarmine and amidoschwarz, only two distinct bands were seen. Eluates of the slower band produced the same effects as crude venom on the various types of muscle described. This fraction moved towards the cathode at both *p*H 2.6 and 8.6. Eluates of the other fractions had no effect on the muscle preparations. It was concluded that the principle active on the muscle preparations is probably protein in nature.

DISCUSSION

The effects of venom, large doses of veratrine, and substances like citrate, which bind calcium ions, were in some respects similar. They all produced a contracture, an initial increase in the amplitude of electrically-induced muscle contractions, and spontaneous twitching. These similarities could be explained by the assumption that the stabilizing function of calcium at the muscle membrane is interfered with by the active constituent of venom.

The subsequent gradual decrease in amplitude of electrically-induced contractions may well be due to fatigue caused by the continual minute twitches and increased tone which were main features of the effect of venom.

Increased concentrations of calcium lessened the effects of venom, and lowered concentrations enhanced them, findings which are in accord with the above hypothesis. When the response of the diaphragm to electrical stimulation was depressed by high concentrations of calcium, it could be restored by adding venom to the bath fluid. Further, in both rat diaphragm and toad sartorius muscle, a contracture could be produced by a combination of a dose of venom, normally subthreshold, and calcium-free Ringer solution.

Two different mechanisms of interference by venom with calcium can be envisaged. Firstly, it may have an action like that of citrate, lowering the concentration of ionized calcium in the surrounding medium by forming an undissociated complex. The active constituent of venom is probably a protein and several proteins are known to form complexes with calcium (Brink, 1954). However, a consideration of the effective doses of venom (2 to 5 μg./ml. of dried crude venom) applied in a calcium concentration of 1.8 mM. indicates that an improbably large number of calcium ions would have to be taken up by each molecule of the venom substance to produce the effects of lack of ionized calcium from the medium. Further, if complex-formation is involved, venom would be expected to produce other effects which resemble calcium-lack, but a concentration of 100 μg./ml. of venom did not interfere with the clotting of blood (Smith, 1958, personal communication). On cardiac muscle the only similarity between the effects of venom and of calcium-lack was a marked decrease in the threshold to electrical stimulation. The lack of effect of venom on the strength of contraction of cardiac muscle would indicate that it did not act at those sites which control the strength of contraction, and for which calcium and sodium compete (Niedergerke and Luttgau, 1957).

The alternative mechanism is a displacement of calcium by venom from the sites that bind calcium at the muscle fibre membrane. A similar explanation of the mode of action of veratrine has been proposed tentatively by Gordon and Welsh, 1948; see also Shanes, 1958.

Differences between the effects of venom and of veratrine have already been mentioned. They consisted mainly in a more rapid depression of the amplitude of electrically-induced contractions and a lack of effect on relaxation by veratrine as compared with venom. In these respects, the effect of veratrine was less like that of calcium-depletion than was the effect of venom. Indeed, various observations, such as the lack of repetitive after-discharge when the whole muscle fibre was exposed to a uniform concentration of veratrine (Burns, Frank, and Salmoiraghi, 1955), and the depression of excitability that occurred after the first depolarization during contact with veratrine (Frank, 1958), are not consistent with the concept that the action of veratrine is due entirely to lack of calcium. Increased excitability and repetitive after-discharges are known to occur when calcium is lacking (Brink, 1954; Shanes, 1958), and it is possible that the mechanical effects of venom observed are associated with similar electrical events. It is obviously necessary to obtain direct measurements of resting potentials and negative after-potentials under the influence of venom before further conclusions on its mode of action can be reached.

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