system was investigated. Radioactive [4-^{14}C]vitamin D_3 (N. V. Philips-Duphar, Amsterdam) was incorporated in the culture medium (80 I.U. of cold D_3 and 320 I.U. of [^{14}C]D_3, 0.4 μc/ml) and incubation was carried out as before. After 48 hours the entire contents of each flask were homogenized and a total lipid extraction performed (*13*). The lipid extract was concentrated by evaporation under a stream of N_2 and an aliquot was applied to a thin-layer plate (Eastman Chromagram), along with D_3 and HCC standards, and developed with 25 percent acetone in *n*-hexane. This system was capable of resolving D_3 ($R_F = 0.53$) and HCC ($R_F = 0.37$) cleanly. The chromatogram was cut into sections that were counted in a liquid scintillation counter. Approximately 2 percent of the total radioactivity remained at the origin, 95 percent migrated in the D_3 section, and the remainder was distributed uniformly from just after the origin through the HCC section at about 1 percent per section. Essentially similar results were obtained with extracts of the medium containing [^{14}C]-vitamin D_3 incubated with no tissue present. Thus, in this organ culture system, there appears to have been little or no conversion of vitamin D_3 to HCC during manifestation of vitamin D_3 action with the possible exception that further conversion of the HCC to some other form, which the chromatographic system was incapable of resolving (*14*), may have occurred. On the basis of these studies, it would seem necessary to consider the possibility that, at least, the vitamin D–responsive system in the intestinal mucosa exhibits a relative, rather than an absolute, specificity toward vitamin D–related sterols.

It is clear that the in vitro organ culture system described represents a unique opportunity to facilitate investigation of many aspects of the mechanism of action of vitamin D_3 on intestinal calcium transport.

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5. R. A. Corradino, A. N. Taylor, R. H. Wasserman, *ibid.*, p. 760. The normal chick embryonated egg contains vitamin D but, for unknown reasons, its intestine does not produce CaBP until after hatching. The in vitro results would suggest that the vitamin D present in ovo may not be accessible to the intestine or that there is some other physiologic mechanism maintaining the intestine in an unresponsive state until after hatching.
6. The purity of this preparation was checked by silicic acid thin-layer chromatography with the use of 25 percent acetone in hexane as developing solvent (vitamin D_3, $R_F = 0.53$; HCC, $R_F = 0.37$). When 10 percent phosphomolybdic acid in 95 percent ethanol was used as a spray reagent (detection limit, 0.25 μg of D_3 or HCC), no trace of HCC was found in a total of 2 mg of vitamin D_3 applied. Thus, in the culturing experiments, no more than 0.05 I.U. of HCC per milliliter could have been present in the medium. At least 5 I.U. of HCC per milliliter are necessary to induce a detectable amount of CaBP.
7. Although the concentrations of vitamin D_3 added to the medium were greater than blood concentrations of vitamin D in intact animals, the response of the embryonic intestine cannot be considered pharmacologic a priori, since the *effective* concentration of D_3 in the medium may be considerably less than the amount added because of insolubility, protein binding, or micelle formation. In addition, a typical physiologic response was obtained with as little as 25 I.U./ml (0.6 μg/ml) of D_3 which can hardly be considered a pharmacologic dose.
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Destruction of Mammalian Motor Nerve Terminals by Black Widow Spider Venom

Abstract. *Black widow spider venom selectively poisons motor nerve endings. A progressive and irreversible failure of neuromuscular transmission occurs in the cat. Electron microscopy of the poisoned nerve-muscle junction shows a sequence of motor nerve ending damage that culminates in disruption of the prejunctional membrane and loss of all organelles, including synaptic vesicles. The postjunctional membrane was morphologically unaffected. After complete poisoning, the contractile response to exogenous acetylcholine was severely impaired, an indication that the prejunctional site is chiefly involved in the contractile response produced by exogenous acetylcholine and that the pre- and postjunctional effects of acetylcholine were separated.*

The venom of the black widow spider (*Latrodectus mactans tredecimguttatus*) destroys amphibian motor nerve endings in what appears to be a highly specific action (*1*, *2*). After application of the venom to the frog nerve-muscle preparation in vitro, there was an enormous increase in the frequency of miniature end-plate potentials (MEPP), after which MEPP frequency declined to low levels. This increase and later decay were interpreted as reflecting a complete discharge of the transmitter stores (*1*). Neuromuscular transmission disappeared at a time when the MEPP frequency had reached a peak and nerve ending depolarization had occurred. Frog neuromuscular junctions that had been exposed to the spider venom were examined by electron microscopy (*2*) and showed a total absence of synaptic vesicles and other organelles; the terminal expansions were evacuated. There could be little doubt that these morphological changes were the cause of the venom-induced, complete and irreversible failure of neuromuscular transmission.

These findings raise the question of whether the spider venom would have a similar selective action on mammalian (cat) motor nerve endings. If there is selective destruction by the venom, then it should be possible to ascertain indirectly the role of the motor nerve endings in the contractile response that is evoked by the intra-arterial injection of acetylcholine (ACh) in innervated muscle in vivo. We had earlier found that intra-arterial ACh depolarizes the motor nerve endings and that the in vivo contractile response of the innervated muscle to ACh is largely a result of this action (*3*–*5*).

To establish the effect of the venom on neuromuscular transmission, the simple in situ nerve-muscle preparation of the cat was used (*3*). The cat was anesthetized with chloralose (80 mg/kg intravenously); the sciatic nerve was sectioned. The soleus nerve and muscle were exposed, and the leg was fixed in

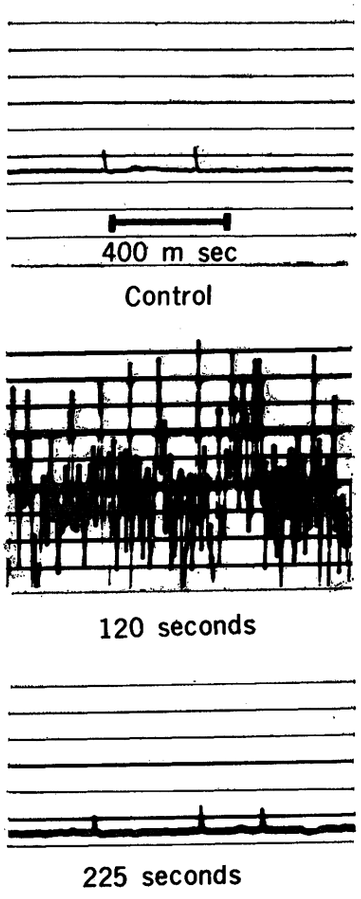

Fig. 1. Effect of black widow spider venom on miniature end-plate potential (MEPP) frequency in cat tenuissimus muscle in vitro (*6*). Times after the addition of the venom (*7*) are indicated in seconds for the representative oscilloscope sweeps. The horizontal time base is the same in all sweeps and is indicated in the control sweep. Horizontal lines are spaced at 200 μv. Experiments were performed at room temperature (24° to 26°C).

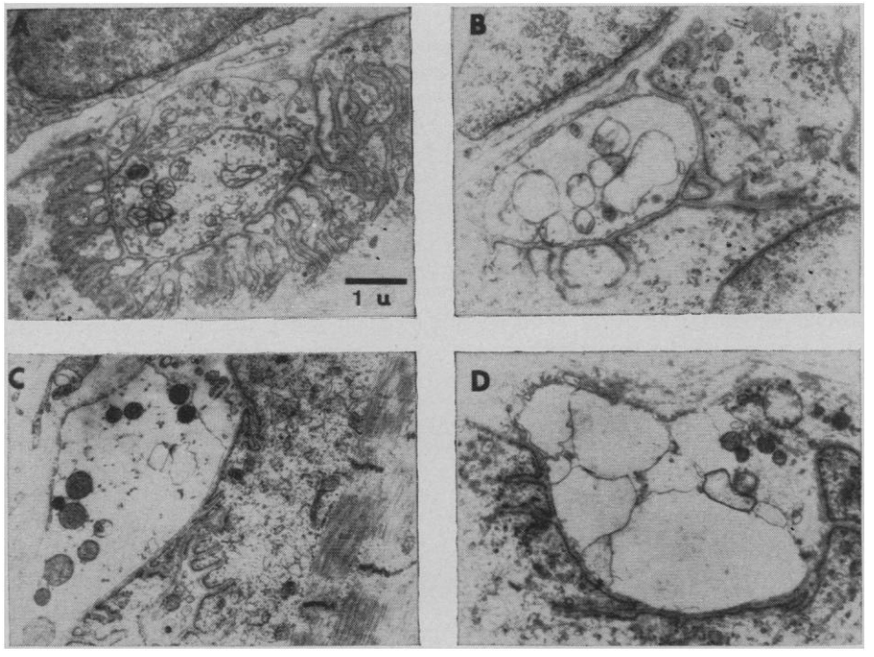

Fig. 2. Electron micrographs showing the effects of black widow spider venom on the morphology of cat motor nerve ending. Sections through neuromuscular junctions of cat soleus muscle. Each section is necessarily from a different animal. The magnification is the same for each (see A). (A) Control, the motor nerve ending lies in the synaptic groove of the muscle. The large Schwann cell covers the neuromuscular junction; (B) 5 minutes after intra-arterial injection of spider venom; (C) 14 minutes after venom injection; (D) 58 minutes after venom injection. Difference in number of postsynaptic infoldings between the control and the experimental models is due to the variation in plane of sectioning and sampling and does not represent a specific process of membrane alteration. The distribution of subsarcolemmal granularity is within normal limits.

a myograph. The calcaneum with the attached tendon was cut free and connected by a steel rod to a strain gauge. The artery to the muscle was exposed for injection. Exposed tissues were covered with mineral oil at 37°C. Isometric contractile responses to single supramaximal nerve stimuli were recorded by a direct-writing oscillograph. A solution of spider venom was obtained by homogenizing four poison glands (from two spiders) in 1 ml of cold physiological saline.

In 20 experiments with the soleus nerve-muscle preparations, the intra-arterial dose of the venom solution that regularly caused failure of neuromuscular transmission was 0.1 ml/kg. Usually 2 minutes after injection of the venom, the twitch began to decline, and one-half of the control height was reached in an average of 6 ± 2.0 (S.E.) minutes; complete block occurred in an average of 16 ± 2.0 (S.E.) minutes. Recovery of neuromuscular transmission was never seen in observation periods extending up to 72 hours in all of five experiments studied. Despite complete neuromuscular block, the muscle always responded normally to direct stimulation. When smaller doses of the spider venom were given, neuromuscular block developed more slowly and was often incomplete. In all cases, whatever block occurred was irreversible. In six separate experiments with the gastrocnemius nerve-muscle preparation the results were identical.

Usually after venom injection and before onset of block, fasciculations and a slight increase (average of 20 percent) in twitch height were seen. Smaller venom doses regularly caused this twitch potentiation. The potentiation and fasciculations probably reflect some depolarization of motor nerve terminals, in accord with the known decrease in spike height in the motor nerve terminal (*1*).

In the recording of MEPP frequency in cat tenuissimus muscle in vitro (*6*), the injection of the venom into the bath (*7*) caused a sharp and large increase in MEPP frequency discharge, just as it does in the frog (*1*). This effect lasted approximately 5 minutes in all experiments (Fig. 1). One interpretation of the MEPP frequency increase is that the spider venom depolarizes motor nerve endings and that this depolarization is presumably a consequence of damage of the presynaptic membrane. It was not possible to measure MEPP amplitude during high frequency discharges (Fig. 1, 120 sec-

onds). However, average MEPP amplitudes measured during control periods and more than 200 seconds after venom administration (Fig. 1) were not different. In five preparations, the resting membrane potential of the muscle was recorded at the end-plate region. The membrane potential did not change for at least 1 hour after the addition of the venom.

The action of the spider venom on cat motor nerve endings was also detected by antidromic monitoring (*3*). Depolarization of unmyelinated motor nerve endings can excite the contiguous myelinated portion of the same axon, leading not only to orthodromic transmission but also to propagated antidromic discharge (*3*, *5*). Thus by antidromic monitoring, it is possible to ascertain indirectly whether spider venom causes motor nerve ending depolarization. By means of a lumbar laminectomy, ventral roots L6 and L7 were disconnected at their points of origin in the spinal cord. Filaments were separated to find one that contained a single functional soleus motor axon; this was identified by stimulation of the soleus nerve in the periphery and recording action potentials from the ventral root filament. Immediately after the intra-arterial injection of the venom (0.1 ml/kg), the single motor axons in 70 percent of the experiments discharged independently of applied stimulation, presumably reflecting depolarization of the endings. Additionally, the motor nerve ending discharges could account for the observed fasciculations. These discharges were never seen after the venom had caused complete neuromuscular transmission block.

We also correlated changes in neuromuscular transmission after venom administration with ultrastructural changes in the nerve endings. Biopsies of the junctional regions of cat soleus muscle were performed with a clamp designed to keep the muscle at normal length during fixation. Samples were fixed for 2 hours in 4 percent formaldehyde in 0.05*M* cacodylate buffer (*p*H 7.2) containing 0.01*M* sucrose. The tissues were then briefly washed in isotonic sucrose, treated with osmium tetroxide, and embedded in Epon 812 after alcoholic dehydration. Thin sections were obtained with an LKB ultramicrotome having a diamond knife and were examined in a Hitachi HS-7 electron microscope at 50 kv.

The electron micrograph of normal cat neuromuscular junction shows the synaptic vesicles and mitochondria within the terminal axonal expansion (Fig. 2A). In the terminal structure 5 minutes after the intra-arterial injection of venom, at a time when the twitch response was 65 percent of control, the prejunctional membrane was intact but the mitochondria showed a loss of cristae and had irregularly laminated outer membranes (Fig. 2B). Also at this time, the synaptic vesicles seemed reduced in number. In a nerve terminal structure 14 minutes after venom injection, when neuromuscular block had just been completed, the prejunctional membrane appeared to be discontinuous, synaptic vesicles were almost completely absent, and the mitochondrial cristae were disrupted (Fig. 2C). After the onset of complete neuromuscular block (58 minutes after venom injection), there was a complete absence of synaptic vesicles and the terminal axonal expansion appeared partitioned into several membrane limited vacuoles (Fig. 2D). Isolated mitochondria and some organelles were pushed to a small corner near the Schwann cell. The prejunctional membrane was destroyed and the postjunctional membrane was lined only by dense basement membrane along the junctional surface.

The initial attack of the venom on the terminals, which is reflected by the great increase in MEPP frequency and the spontaneous discharging of motor axons, occurred prior to the severe morphological damage. Examination of muscle structures including postjunctional subsarcolemmal organelles, myofibrils, sarcomeres, sarcoplasmic reticulum, transverse tubules, and sarcolemmal nuclei disclosed no significant abnormalities. Thus the ultimate block of response to indirect stimulation with preservation of the direct response accords with the selective striking destruction of the motor nerve terminals.

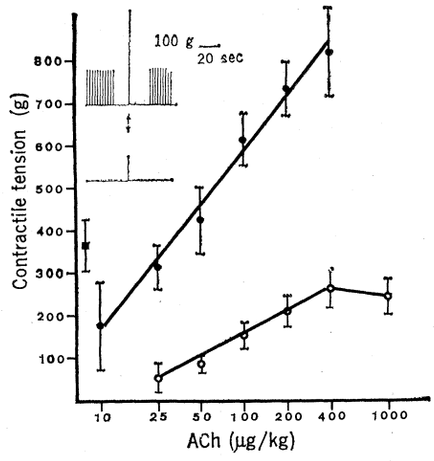

Fig. 3. Dose-response analysis of the effect of black widow spider venom on the isometric contractile responses of cat soleus muscle to intra-arterially injected acetylcholine (ACh). Mean response (± S.E.) evoked by ACh doses before (filled circles) and after (open circles) destruction of motor nerve endings by the venom. Each point was determined by at least six experiments; each linear regression was calculated by the method of least squares. For comparison, the isolated filled square to the left represents, from 20 experiments, the mean isometric tension (± S.E.) developed by single, supramaximal stimulation of motor nerve prior to poisoning. The insets show typical muscle responses to nerve stimulation and (at arrow) to an ACh dose of 200 μg/kg before (upper) and after (lower) motor nerve ending destruction by the venom. The ACh response after poisoning is not only less than in the control but is also less than the contractions normally elicited by single, supramaximal stimulations of the motor nerve (upper trace).

The influence of the venom injection on postjunctional effects of ACh was evaluated in cat soleus muscle that was surgically denervated by section of the sciatic nerve at the level of the hip 6 days prior to experiment. Isometric contractile responses to varying intra-arterial ACh doses were determined in this in situ preparation. The data from six experiments showed no difference between the contractile responses to ACh given before and after venom administration. From these identical dose-response relationships, we concluded that the venom does not affect the reactivity of the postjunctional membrane to ACh. This finding accords with the failure of the venom to alter the morphology of the muscle membrane, its resting potential, or the MEPP amplitude.

In the normally innervated nerve-muscle preparation, shortly after venom injection, when twitch height was still more than one-half of the control, ACh usually evoked little or no contractile response. Long after neuromuscular block was complete (1 hour) and presumably when motor nerve endings were totally destroyed, a dose-response analysis of ACh was made (Fig. 3). For intra-arterial doses (10 to 1000 μg/kg), the curve after venom poisoning as compared with that of the control is flattened and shifted to the right. In view of the motor nerve ending destruction, there can be little doubt that the difference in the dose-response regressions before and after venom injection reveal the important role of

motor nerve endings in response to exogenous ACh.

The effect of venomous impairment on the action of injected ACh in the innervated neuromuscular junction was disclosed more directly in three experiments by antidromic monitoring. We found that the motor nerve discharges which ordinarily follow intra-arterial ACh injection (*3*) were uniformly absent. Because this ACh-evoked neural activity arises in the nerve ending, the absence of firing after venom injection is not unexpected in view of the motor nerve terminal destruction described above.

The limited ACh contractile responses that remain after venom poisoning (Fig. 3) likely express the reactivity of the postjunctional membrane to ACh. This dose-response relationship is, we think, an unequivocal quantitative description of postjunctional reactivity to ACh. The destruction of the motor nerve terminals has eliminated the large neural component in this response, and denervation hypersensitivity is not yet present. Furthermore, the post-venom dose-response relationship (Fig. 3) shows that no matter how large the ACh dose (including doses up to 1 mg/kg) the contractile response never exceeds the twitch height, as is regularly the case in the normal system when doses of 50 μg/kg or larger are given. Therefore the much greater responses of the normal muscle must reflect not only the synchronizing influence of the widespread ACh evoked motor nerve terminal discharge but also the repetitive nature of that response. Thus, selective poisoning of motor nerve terminals by spider venom confirms in yet another way, the conclusions reached in earlier studies from this laboratory, wherein it was shown by two distinctly different methods, namely antidromic monitoring (*3*) and subacute denervation (*4*), that the motor nerve terminals are the major site of exogenous ACh action.

Because of the apparently selective and irreversible action of black widow spider venom on motor nerve endings, there is at hand a unique means by which the functional and pharmacologic properties of these important nerve endings may be explored and related to fine structure. However, if the full potential of this reagent is to be realized, it will be necessary to isolate the active principle. This would enable, through dose-response analysis, a full and precise definition of its selectivity at the neuromuscular complex.

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L-Glutaminase: Suppression of Lymphocyte Blastogenic Responses in vitro

Abstract. *Continuous exposure to 0.1 international unit or more of* Escherichia coli L-*glutaminase inhibited responses of human lymphocytes to phytohemagglutinin, streptolysin O, and allogeneic leukocytes. Inhibition was completely reversed by removing the enzyme from the culture or adding* L-*glutamine but not* L-*asparagine. Cytoxicity did not occur.* L-*Glutaminase should be immunosuppressive in vivo.*

In the search for new immunosuppressive agents, the effects of the enzyme L-glutaminase on the in vitro blastogenic responses of human peripheral blood lymphocytes to mitogen stimulation were investigated. It was hypothesized that L-glutaminase, which catalyzes the conversion of L-glutamine to L-glutamic acid, might be immunosuppressive for the following reasons. Glutamine antagonists such as azotomycin are immunosuppressive (*1*). Although L-glutamine is not an essential amino acid, lymphoid tissue contains a small amount of glutamine-synthetase (*2*) and gets its L-glutamine from the blood (*3*). Some cells have a population-dependent requirement for L-glutamine in vitro (*4*). Experimental lymphoid malignancies are susceptible to the antitumor action of L-glutaminase (*5*), and specific enzyme-induced amino acid deficiency is immunosuppressive in the case of L-asparaginase (*6*) which may contain 1 to 3 percent glutaminase activity.

The lymphocyte culture system was selected for initial studies of this enzyme because it has provided significant data on the function of the human lymphoid system (*7*) and has been useful in evaluating the mechanisms of action of immunosuppressive drugs such as azotomycin (*1*) and L-asparaginase (*6*). Cultures were set up according to described methods (*8*). Each tube (13 by 100 mm) contained 4×10^5 lymphocytes, in a medium composed of 0.3 ml of autochthonous serum, 0.7 ml of L-glutamine–free and L-asparagine–free minimal essential medium (Spinner-modified), and 100 units of penicillin and 100 μg of streptomycin per milliliter. Cultures were stimulated with one of the following mitogens: 0.02 ml of phytohemagglutinin (PHA), 0.03 ml of streptolysin O (SLO), or 4×10^5 allogeneic lymphocytes previously irradiated with 5000 rads (WBC) (*9*). *Escherichia coli* L-glutaminase (*9*) was dissolved in saline, kept at 4°C and used within 2 weeks. L-Glutamine was supplied as a 200 m*M* solution, was stored frozen and thawed only once before use. L-Asparagine (*9*) was dissolved in saline, frozen only once, and thawed before use. These cultures were incubated at 37°C for 3, 5, and 7 days, respectively, and then incubated for 2 hours in the presence of 2 μc of tritiated thymidine (specific activity, 1.9 c/mmole). Acid-insoluble radioactivity was measured by liquid-scintillation counting (count/min per 4×10^5 lymphocytes). Slide preparations were also made and the percentages of lymphoblasts in 500 cell counts were recorded.

L-Glutaminase, when present from the beginning of the culture period, was a potent inhibitor of blastogenesis in vitro (Table 1). Doses of 0.01 I.U./ml were significantly inhibitory. Doses of 0.1 I.U./ml inhibited thymidine incorporation approximately 87 to 95 percent