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THE EFFECTS OF THE SUBCUTANEOUS INJECTION OF THE CRUDE VENOM OF THE AUSTRALIAN COMMON BROWN SNAKE, *Pseudonaja textilis* ON THE SKELETAL NEUROMUSCULAR SYSTEM

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1 The effects of the crude venom of the Australian common brown snake on the mammalian neuromuscular system have been investigated.

2 The venom was injected subcutaneously into the dorso-lateral aspect of one hind limb of the rat. The limb was paralysed within 90 min and remained paralysed for 2 to 3 days.

3 The exposed muscles failed to respond to indirect excitation, and individual fibres were not depolarized at the nerve-muscle junction by exposure to carbachol.

4 The wet weight, histological appearance, resting potential and input resistance of the muscle fibres and their ability to generate directly elicited action potentials were unaffected by exposure to the venom.

5 Administration of venom to isolated preparations caused a reduction in the amplitude of miniature endplate potentials, with no change in frequency. The quantal content of evoked endplate potentials was unchanged.

6 It was concluded that the crude venom was largely devoid of presynaptic activity and myotoxicity, and that its primary site of neurotoxicity was directed to the postsynaptic membrane.

Introduction

The common brown snake, *Pseudonaja textilis*, is an elapid snake widespread throughout Queensland, Victoria, New South Wales and south-eastern parts of South Australia. It is closely related to two other Australian elapids, the collared brown snake (*Pseudonaja nuchalis nuchalis*) and to the spotted brown snake (*Pseudonaja nuchalis affinis*). All three are large, easily aroused, dangerous snakes and their respective venoms are considered powerfully neurotoxic (Garnet, 1977).

Recent observations on the biological activity of the venoms of several Australian elapid snakes have shown that in addition to being neurotoxic, they are myotoxic. To date, such activity has been determined in the venoms of the king brown snake (*Pseudechis australis*), the tiger snake (*Notechis scutatus*) and the taipan (*Oxyuranus scutellatus*), and in the case of *Pseudechis australis* and *Notechis scutatus* the myotoxic activity is now considered to be of clinical importance (Rowlands, Mastaglia, Kakulas & Hainsworth, 1969; Sutherland & Coulter, 1977). These observations have prompted a reinvestigation of the biological activity on the neuromuscular system of the crude venom of *Pseudonaja textilis*.

Methods

Most experiments were performed on soleus muscles isolated from female Wistar rats weighing 160 to 180 g, although hemidiaphragm muscles were occasionally used. In most experiments a single injection of 2.0 μg (0.2 ml of 10 μg/ml in 0.9% w/v NaCl solution) of the crude venom was made subcutaneously into the dorso-lateral aspect of one hind limb at the line of demarcation between gastrocnemius and soleus. The injection was so directed that venom was administered into the vicinity of the soleus muscle, rather than directly into the muscle, thus avoiding mechanical damage to the fibres. Control injections of 0.2 ml of 0.9% w/v NaCl solution (saline) did not have any effect on either the morphology or the physiological behaviour of the muscle. At various times after the injection of the venom the soleus muscles from both the injected and the contralateral limbs were removed for histological or physiological examination.

In other experiments, crude venom was added to the solution bathing an isolated muscle. The conditions of these various experiments are identified in the text.

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Histology

Blocks of tissue comprising the middle 5 mm of the soleus muscles were sandwiched between thin slices of liver and orientated so that transverse sections could be obtained. The tissue blocks were then frozen in dichlorodifluoromethane (ICI Ltd.) maintained at about −150°C in liquid nitrogen. Transverse sections, 10 μm thick, were stained with haematoxylin and eosin and mounted in the usual manner.

Physiological bathing fluids

The isolated muscles were bathed in a fluid of the following composition (mM): K^+ 5.0, Na^+ 150, Ca^{2+} 2.0, Mg^{2+} 1.0, Cl^- 148, $H_2PO_4^-$ 1.0, HCO_3^- 12.0, and glucose 11.0. The solution was maintained at room temperature and equilibrated with 5% CO_2 and 95% O_2.

Electrophysiological recording methods

The passive and active electrical responses of muscle fibres were recorded with glass microelectrodes filled with 3 M KCl. The electrodes had tip potentials less than 5 mV and resistances of 5 to 15 MΩ. A second microelectrode, inserted into a muscle fibre 50 to 100 μm away from the recording electrode, was used to pass current of the appropriate polarity when it was necessary to measure 'input resistance' or to generate 'direct' action potentials from a standardized resting potential of −90 to −95 mV (as described by Redfern & Thesleff, 1971a, b).

Endplate potentials

Endplate potentials (e.p.ps) were recorded from preparations partially blocked by exposure to a high concentration of Mg^{2+} (15 mM). They were generated by stimulating the motor nerve with pulses of 0.05 ms duration at a supramaximal intensity and a frequency of 0.1 Hz. At this frequency there was no measurable early rundown of e.p.p. amplitude. The amplitude of the first 10 e.p.ps and of the m.e.p.ps occurring over the same period was measured. The quantum content of the e.p.p. was calculated from the ratio mean e.p.p. amplitude: mean m.e.p.p. amplitude. E.p.p. amplitudes were not corrected for non-linear summation since they seldom exceeded 8 mV; at such amplitudes, the correction factor is negligible (Martin, 1976).

Experiments utilizing the twitch response

These experiments were carred out on isolated hemidiaphragm preparations. The phrenic nerve was stimulated at 0.1 Hz with pulses of 0.05 ms duration and supramaximal voltage. The contractions were recorded isometrically with a Devices Ltd. dynamometer (UF1), amplifier (3559; HF cut 150 Hz) and recorder (MXZ).

Presentation of results

The results are routinely presented as mean ± s.e. mean. The difference between the two means was tested using Student's *t* test. A probability level of $P<0.05$ was considered statistically significant.

Results

Experiments on muscle from venom-treated animals

Condition of animals Within 60 to 90 min of the injection of venom, the envenomed leg was paralysed and it remained paralysed for about two days. During this time there was no evidence of a generalized neuromuscular disorder, and no unusual lachrymation or salivation. The animals moved freely and fed and groomed normally. It was concluded that the effects of the venom were localized to the injected limb and that muscles from the contralateral limb could serve as controls.

Effects of the venom on muscle Muscles removed 24 h after the injection of venom exhibited no obvious evidence of damage and no oedema. Frozen sections of the muscles (10 μm thick, stained with haematoxylin and eosin) appeared normal. The

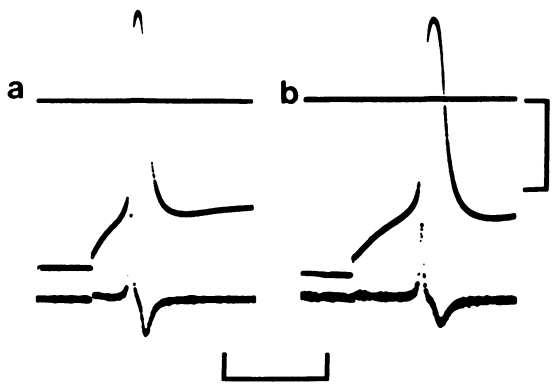

Figure 1 Directly elicited muscle fibre action potentials generated using a double microelectrode technique from a preset membrane potential of −90 mV. (a) Soleus muscle isolated 24 h after injection of 2.0 μg *Pseudonaja textilis* venom; (b) contralateral soleus muscle. Note that although the injected muscle was paralysed, it generated action potentials of normal configuration. Upper trace: zero potential; middle trace: voltage record; lower trace: 1st derivative of voltage record. Calibration: vertical, 50 mV, 300 V/s; horizontal, 5 ms.

Table 1 Some properties of muscle fibres of rat soleus after injection of the venom of *Pseudonaja textilis*

	Contralateral muscles	*Envenomed muscles*
Muscle wet weight: mg	147 ± 2 (6)	143 ± 4 (6)
Membrane potential: mV	−72 ± 0.3 (271)	−71 ± 0.7 (67)
Input resistance: MΩ	0.36 ± 0.029 (16)	0.37 ± 0.017 (17)
Action potential threshold: mV	−54 ± 0.5 (31)	−56 ± 0.9* (10)
Action potential overshoot: mV	25 ± 1.0 (31)	22 ± 1.4 (10)
Action potential 1st derivative: V/s	306 ± 14 (31)	359 ± 12* (10)

The venom (2.0 μg in 0.2 ml saline) was injected into one hind limb. Both soleus muscles were removed 24 h later. Action potentials were generated using a double microelectrode technique from a resting potential artificially preset to a level of −90 mV. The results are expressed as mean ± s.e. and the numbers of observations are given in parentheses.

*Difference between the means significant at 5% level.

mean muscle wet weights were indistinguishable from normal, and so were the mean muscle fibre resting membrane potentials and input resistances (Table 1). Action potentials of normal configuration (Figure 1) could be generated at will using the double microelectrode technique for direct stimulation (see Methods). The threshold for action potential generation in the envenomed muscles was more negative than normal, and the maximum rate of rise was higher than normal (Table 1). However, although these differences were statistically significant they were so small that their biological importance is probably negligible.

Action potential generation in muscle fibres following nerve stimulation Indirect action potentials could be generated routinely in contralateral muscle fibres. However, within 60 min, 30% of superficial fibres in muscles removed from the envenomed limb failed to generate an action potential and by 90 min all fibres were inexcitable. The superficial fibres remained paralysed for a relatively short period. Thus, by two days, 50% of muscle fibres were excitable again and by three days functional reinnervation was virtually complete (Figure 2). At this stage, the indirectly elicited action potentials had a normal configuration.

The inability of muscle fibres to generate an action potential in response to nerve stimulation was not due to a loss of membrane potential or to a change in the threshold for action potential generation to a more positive value because at 24 h, when all the muscle fibres were inexcitable, the mean resting membrane potential was indistinguishable from normal and action potential thresholds were more negative than normal (Table 1).

The effects of carbachol on muscles removed from envenomed animals Envenomed and contralateral control muscles were removed from two rats 90 min after the injection of *Pseudonaja textilis* venom. Muscle fibre resting membrane potentials were measured at the endplate region before and 2 min after the addition of carbachol (5×10^{-5}M) to the bathing fluid. The muscle fibres of envenomed muscles were depolarized by 2 and 13 mV respectively compared with the 'normal' depolarization of 22 and 23 mV respectively (Table 2).

The effects of venom administration to isolated nerve-muscle preparations

The indirect twitch response of the diaphragm Venom (5 to 10 μg/ml) caused neuromuscular failure in the isolated hemidiaphragm preparation. In three preparations exposed to 10 μg/ml venom the time to 50% blockage was 30, 34 and 40 min respectively, and the block was complete by 60 min (Figure 3). The progress of the block was unaffected by the administration of neostigmine (3×10^{-6}M).

In three other experiments, Mg^{2+} (12 mM) was used to cause a partial (≏50%) block of neuromuscular transmission. In these preparations, the administration of venom (5 or 10 μg/ml) caused neither a transient nor a long lasting alleviation of the Mg^{2+}-induced block; instead the depth of block was enhanced.

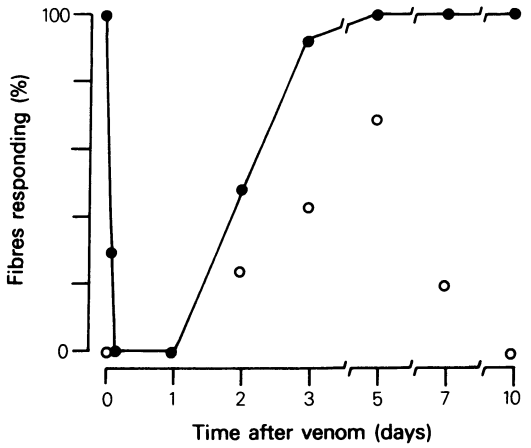

Figure 2 The loss and restoration of functional innervation (●) and the appearance of tetrodotoxin resistant action potentials (O) in soleus muscles as the result of the subcutaneous injection of 2.0 μg *Pseudonaja textilis* venom. Each point is calculated from observations on 20 to 150 individual fibres. The data on 'innervation' are presented as the percentage of fibres sampled that responded to indirect excitation by generating an action potential. The data on 'tetrodotoxin-resistance' are presented as the percentage of fibres generating an action potential in the presence of tetrodotoxin following direct stimulation, using the double microelectrode technique, from a preset membrane potential of −90 mV.

Pseudonaja textilis antivenom (1.25 units/ml) did not reverse the block caused by venom, but 10 min pre-incubation with the same concentration of antivenom protected the nerve-muscle preparation against the venom (Figure 3).

Antivenom alone had no effect on transmission in normal preparations. In partially blocked preparations (either Mg^{2+} 12 mM to 15 mM or (+)-tubocurarine 10^{-6} M), antivenom caused a small (5 to 10%), transient alleviation of the block, but this was shown to be due to the presence of cresol in the antivenom preparation (see also Datyner & Gage, 1973).

Resting membrane potential Exposure of isolated soleus muscles to venom (5.0 μg/ml) for 45 min caused no significant reduction of the resting membrane potential (control -74 ± 0.8 mV; $n = 24$; test -71 ± 1.8 mV; $n = 17$). The various parameters of the action potentials were not measured, but they did not appear to differ from those generated before exposure to venom.

Miniature endplate potentials After the addition of venom (5 to 10 μg/ml) miniature endplate potentials (m.e.p.ps) could be recorded from superficial fibres for approximately 10 to 15 min. During this time they declined steadily in amplitude, but without change in frequency. A typical experiment in which m.e.p.ps were recorded continuously for 15 min is summarized in Table 3. It may be seen that m.e.p.p. amplitude declined from a mean of 200 μV to a barely detectable level ($\simeq 50$ μV) between 12 and 15 min after the addition of venom (5.0 μg/ml).

Two muscles exposed to venom (5.0 μg/ml) for 15 min were subsequently exposed to carbachol (5×10^{-5} M). The mean membrane potentials recorded at the endplate regions of 15 to 20 muscle fibres differed from controls by +1.1 mV and −0.8 mV respectively.

Endplate potentials Endplate potentials (e.p.ps) were generated in response to nerve stimulation at a rate of 0.1 Hz. The quantum content of the e.p.ps, calculated by the direct method before the administration of *Pseudonaja textilis* venom, was 8.2 ± 0.75 ($n = 20$). After exposure for 10 min to *Pseudonaja textilis* venom, 1.0 or 2.5 μg/ml, the quantum content was 8.1 ± 1.65 ($n = 11$) and 7.0 ± 0.56 ($n = 15$) respectively. The effect of the venom was not statistically significant.

Table 2 The effect of carbachol, 5×10^{-5} M, on the resting potential of the endplate region of muscle fibres in contralateral and envenomed soleus muscles of the rat

	Rat 1		*Rat 2*	
	Resting potential: (mV)		*Resting potential:* (mV)	
	Contralateral	*Envenomed*	*Contralateral*	*Envenomed*
Before carbachol	-73 ± 1.6 (11)	-73 ± 1.0 (10)	-74 ± 1.4 (11)	-71 ± 0.6 (14)
After carbachol	-50 ± 3.3 (10)	-61 ± 2.3 (10)	-52 ± 1.1 (23)	-69 ± 0.8 (27)
Depolarization: mV	23	12	22	2

Carbachol was applied directly to the isolated muscles which had been removed from the rats 90 min after venom injection. The results are expressed as mean ± s.e. and the numbers of fibres sampled are given in parentheses.

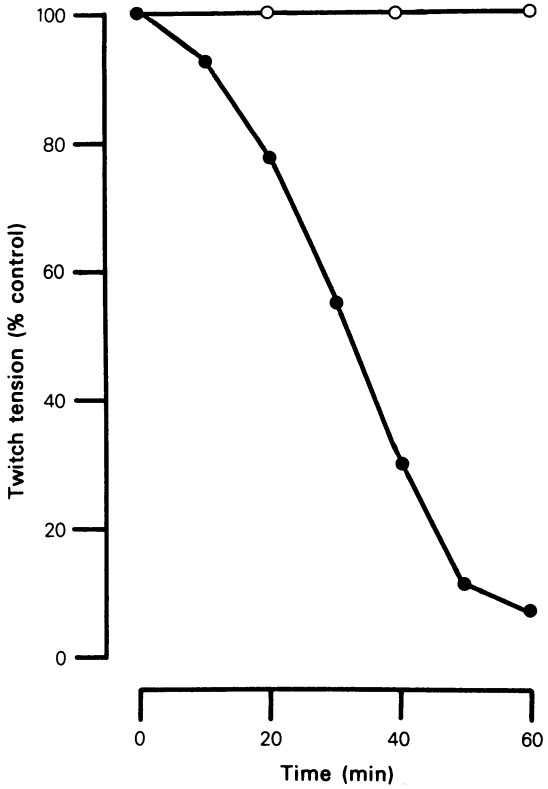

Figure 3 The effect of *Pseudonaja textilis* venom (10 μg/ml) on the twitch tension of the rat isolated hemidiaphragm (●). Muscles preincubated with specific antivenom, 1.25 units/ml were unaffected by the venom (○).

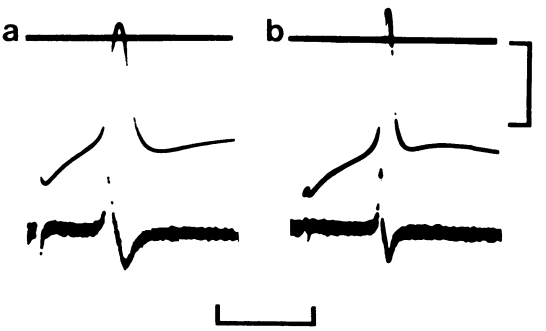

Figure 4 Tetrodotoxin-resistant action potentials recorded from soleus muscle fibres 3 days (a) and 5 days (b) after the injection of 2.0 μg *Pseudonaja textilis* venom. The action potentials were generated using the double microelectrode technique from a present membrane potential of −90 mV. Upper trace: zero potential; middle trace; voltage record; lower trace: 1st derivative of voltage record. Calibration: vertical, 50 mV, 150 V/s; horizontal, 5 ms.

Induction of tetrodotoxin resistant action potentials in muscles of envenomed rats

Muscles removed from envenomed rats were routinely examined for their excitability following nerve stimulation, and then for their ability to generate direct action potentials in the presence of tetrodotoxin (TTX, 10^{-6}M). The results (presented in Figure 2) demonstrated that by two days after venom injection 24% of all fibres tested were able to generate TTX-resistant action potentials, and that this proportion rose to a maximal level of 71% by five days. Some typical tetrodotoxin-resistant action potentials are shown in Figure 4. The number of fibres that were TTX-resistant declined rapidly thereafter, to 20% at day seven. The muscle fibres were normal with respect to TTX, 10^{-6}M, 10 days after envenomation.

Table 3 The effect of *Pseudonaja textilis* venom (5.0 μg/ml) on m.e.p.ps recorded from a soleus muscle fibre

Time (min)	*Membrane potential* (mV)	*M.e.p.p. amplitude* (μV)	*M.e.p.p. frequency* (per s)
0 – 0.5	−72	200	1.9
2 – 2.5	−70	194	2.1
4 – 4.5	−69	135	1.9
6 – 6.5	−67	135	1.8
8.5 – 9.0	−63	123	1.8
12 – 12.5	−62	50	1.3
15 – 15.5	−62	Could not detect m.e.p.ps	

The muscle fibre was impaled throughout the recording period. All m.e.p.ps occurring during the stated time periods were collected. Amplitudes were corrected to a resting potential of −72 mV. Note that there was no measurable change in frequency until about 12 min when amplitude was so low that it was not always easy to detect a m.e.p.p. in the baseline noise. The apparent change in resting membrane potential during the experiment was a consequence of the prolonged insertion of the microelectrode rather than of exposure to the venom *per se*.

Discussion

The administration by injection of the crude venom of the Australian common brown snake had no effect on either the histological appearance or wet weight of the rat soleus muscle, and (with the exception of the development of TTX resistance) had little or no effect on the physiological properties of individual muscle fibres. Since the doses injected were sufficient to cause clinical evidence of neuromuscular paralysis, it seems improbable that the venom has any significant myotoxic activity. In this respect, it is of interest that at similar doses, the crude venom of the Australian elapid snakes *Pseudechis australis*, *Notechis scutatus* and *Oxyuranus scutellatus* cause severe muscle damage, demonstrable as oedema, muscle fibre degeneration and loss of excitability within 3 to 6 h of injection (Rowlands *et al.*, 1969; Harris, Johnson & Karlsson, 1975; Harris, Johnson & MacDonell, 1977; Harris & Johnson, 1978).

The soleus muscles of the envenomed legs were inexcitable when stimulation via the soleus nerve was attempted. The onset of paralysis was rapid, occurring within 90 min, and lasted for 1.5 to 3 days. Since the paralysed muscle fibres were capable of generating action potentials when stimulated directly, the site of venom-induced paralysis was identified as the post-junctional membrane, the motor nerve terminal or the motor nerve axon.

There was clear evidence of a major involvement of the postjunctional membrane. This evidence may be summarized as follows. There was a reduction in the amplitude of m.e.p.ps without a change in frequency, and the depolarizing activity of carbachol at the neuromuscular junction was inhibited after either the *in vivo* or *in vitro* administration of the venom.

Unlike the venoms of the tiger snake, *Notechis scutatus* and the taipan, *Oxyuranus scutellatus* which have clearly demonstrated presynaptic activity (Datyner & Gage, 1973; Harris *et al.*, 1973; Kamenskaya & Thesleff, 1974) no evidence was found for a major action of common brown snake venom on the motor nerve terminal. Thus, at low doses (i.e. below 5 μg/ml) the venom had no effect on evoked transmitter release, whether measured in terms of the indirect twitch-response of partially curarized or Mg^{2+}-poisoned preparations, or in terms of the quantum content of e.p.ps. At higher doses (5 to 10 μg/ml), when transmission was blocked in superficial fibres within 15 min, neuromuscular failure was never accompanied by any changes in m.e.p.p. frequency.

The apparent lack of myotoxicity of the venom has been confirmed in the monkey by Sutherland & Campbell (1980) but the apparent lack of presynaptic activity is possibly contentious. Coulter, Broad & Sutherland (1980) have isolated a high molecular weight protein (textilon) from the crude venom of *Pseudonaja textilis*, and preliminary studies by Southcott & Coulter (1980) have suggested that it is a presynaptically active neurotoxin. If this is so, the apparent lack of such activity in the crude venom presumably reflects the overriding potency of the postsynaptically active fractions. It seems of some interest to characterize fully the biological activity of textilon in view of the observation that in other Australian elapidae, the presynaptically active neurotoxins (that is, notexin, notechis II-5 and taipoxin) are all very potent myotoxins; β-bungarotoxin, one of the toxins isolated from the venom of the Asian elapid, *Bungarus multicinctus*, is the only presynaptically active snake venom neurotoxin to be devoid of demonstrable myotoxicity so far identified (Harris, Johnson & MacDonell, 1980).

It is generally accepted that tetrodotoxin-resistant action potentials in mammalian skeletal muscle fibres arise as a response to denervation or inactivity (Thesleff, 1979). Grampp, Harris & Thesleff (1972) showed that the *in vivo* administration of actinomycin D, an inhibitor of RNA synthesis, inhibits the appearance of tetrodotoxin resistance in denervated mouse muscle only if given during the first two days of denervation. Lømo & Slater (1978) have shown that the denervation induced increase in acetylcholine sensitivity, that parallels the appearance of tetrodotoxin resistance (Harris & Thesleff, 1971), may be inhibited by the direct stimulation of a muscle provided the stimulation starts within the first two days of denervation. It seems of some interest in this context that following the *in vivo* administration of the venom of *Pseudonaja textilis*, the superficial muscle fibres remained paralysed for a maximum of 1.5 to 3 days, and that the number of fibres exhibiting tetrodotoxin-resistant action potentials increased steadily from 24% at day 2 to 71% at 5 days.

All of the muscle fibres in the envenomed soleus muscles appeared to be functionally innervated by between 3 and 5 days; they became 'normally' sensitive to tetrodotoxin between 7 and 10 days. Thus, there was a delay of some 4 to 6 days between the re-establishment of full, functional innervation and the restoration of 'normal' pharmacological behaviour. This is very similar to the 5 day period reported by Løme & Slater (1978) as being the duration needed for imposed activity to reverse a denervation-induced increase in acetylcholine sensitivity in rat soleus muscle fibres.

There is no information on the structure of the postsynaptically active component(s) of *Pseudonaja textilis* venom, but its identification could be of value to those interested in the trophic interactions between a peripheral motor nerve and a skeletal muscle fibre in view of its characteristic duration of action and its ability to cause localized paralysis at the site of injection.

In conclusion, a study has been made of some of the properties of the crude venom of the Australian elapid *Pseudonaja textilis*. The venom appears to be devoid of notable presynaptic neurotoxicity or myotoxicity. It contains a (some) fraction(s) capable of initiating a postsynaptic failure of neuromuscular transmission which lasts for 2 to 3 days.

The crude venom of *Pseudonaja textilis* was obtained from Mr Eric Worrel, Australian Reptile Park, Gosford, N.S.W. Australia.

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