PARALYTIC EFFECTS OF "PARALYTIC SHELLFISH POISON" ON FROG NERVE AND MUSCLE

BY

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A purified extract of toxic lamellibranchs, *Saxidomus giganteus* (Deshayes), containing "paralytic shellfish poison," has been tested for its effects on conduction and contraction in frog nerve and muscle. The poison was very toxic and concentrations within the range 0.025 to 0.1 μg/ml. paralysed isolated muscle preparations, with abolition of the muscle action potential. The poison did not readily penetrate the perineurium, but in desheathed sciatic nerves the conduction of nerve impulses was rapidly blocked by concentrations of 0.05 to 0.1 μg/ml. There was no evidence that the poison had any specific curarizing action at the neuromuscular junction, and the paralysis was not accompanied by any appreciable depolarization of the muscle membrane.

At certain seasons of the year some bivalve molluscs, especially mussels (*Mytilus*) and clams (*Saxidomus*), may become toxic to humans and fatalities occasionally occur. The aetiology of these outbreaks of poisoning has been studied by Meyer, Sommer and their co-workers (Sommer & Meyer, 1937 ; Sommer, Whedon, Kofoid & Stohler, 1937 ; Meyer, 1953), and more recently by Schantz (1960, 1963) and his colleagues. The poison of the lamellibranchs originates in plankton dinoflagellates (*Gonyaulax catenella*, Whedon and Kofoid) ingested by the molluscs which remain toxic for some time. In man the symptoms which follow the eating of these toxic shellfish revolve mainly around various sensory disturbances, including paraesthesias, and may also involve muscular paralysis (Seven, 1958).

Recently the poison has been available as a purified and highly concentrated extract. The empirical formula of the hydrochloride has been established as $C_{10}H_{17}N_7O_4.2HCl$ but little is known either of its chemical structure or of its effects upon the nervous system and muscles (Schantz, 1960). The work described here was undertaken to help in clarifying the site of action of the toxin, and to remove some of the uncertainties that have emerged from previously published studies of the physiological and pharmacological effects of the poison (Kellaway, 1935 ; Covell & Whedon, 1937 ; Fingerman, Forester & Stover, 1953 ; Sapeika, 1953 ; Bolton, Bergner, O'Neill & Wagley, 1959 ; D'Aguanno, 1959 ; Murtha, 1960 ; Pepler & Loubser, 1960).

METHODS

The paralytic shellfish poison was obtained from Dr E. J. Schantz as a purified extract from toxic Alaskan butter clams *Saxidomus giganteus* (Deshayes) (lot 3, no. 69), dissolved at a concentration of 100 μg/ml. in 0.1 N-hydrochloric acid containing 15% ethanol (Schantz, McFarren, Schafer & Lewis, 1958). A stock solution (10 μg/ml.) was prepared by diluting the extract with distilled water and was kept in the refrigerator. It did not lose potency as tested by the mouse median death time (Schantz *et al.*, 1958) during the few weeks storage. For use, portions of this stock solution were diluted with an equal volume of double strength, bicarbonate-free, frog Ringer solution and brought to *p*H 7 with 0.1 N-sodium hydroxide solution, using bromothymol blue indicator.

For further dilution of the poison, and for general use with frog isolated nerve and muscle preparations, Krebs solution was modified to the following constitutions (g/l.): NaCl 5.05, KCl 0.16, $CaCl_2$ 0.20, $MgSO_4$ $7H_2O$ 0.15, $NaHCO_3$ 2.0, KH_2PO_4 0.20 and glucose 1.0. This solution was bubbled with 5% carbon dioxide in oxygen to keep the *p*H near 7.3. On a few occasions when bubbling was inconvenient a bicarbonate-free Ringer solution was used, buffered to *p*H 7.3 with Na_2HPO_4 0.23 g/l. and $NaH_2PO_4.2H_2O$ 0.07 g/l., and with glucose (1 g/l.) added.

All the experiments were done on nerves or muscles from *Rana temporaria* L., with the exception of one experiment on the toad sartorius muscle preparation. The preparations were kept at room temperature (18 to 25° C).

Gastrocnemius muscle contractions were recorded on smoked paper by an isotonic lever giving a magnification of thirteen-times. The electrical stimuli were pulses of 0.1 to 0.5 msec duration, at a supramaximal voltage that varied from one preparation to another. Stimuli were delivered and action potentials led off to cathode followers by platinum wire-electrodes. Action potentials were photographed from an oscilloscope after amplification.

Muscle injury potentials were studied by crushing the insertion end of an isolated sartorius muscle and leading off the d.c. potential between a silver-silver chloride electrode at this (negative) end and another similar electrode which either rested on the uninjured surface near the middle of the muscle or which dipped into the Ringer solution which bathed the proximal half of the muscle and pelvis.

Other drugs used were D-tubocurarine (Burroughs Wellcome, Tubarine Miscible) and cobra venom (Light & Co., *Naja naja* SN33p).

RESULTS

Toxicity determinations

Occasional checks were made on the toxicity of the stock solutions, using the method of Schantz *et al.* (1958). One ml. of a solution containing 0.30 μg/ml. of poison, when injected intraperitoneally into mice of 19.5 to 23.2 g weight, caused death in 5.75 to 8 min. If a correction was made for body weight the median death time indicated a potency not significantly lower than the 5.5×10^{-6} mouse units/g claimed for the pure poison (Schantz *et al.*, 1958).

Frogs, on the other hand, were much more resistant to the effect of the poison whether it was given orally, intraperitoneally or subcutaneously into the dorsal lymph spaces. A dose of 1 to 2 μg of the poison could be injected into frogs (20 to 25 g) without causing death.

Nerve-muscle preparation

When a sciatic nerve and gastrocnemius muscle preparation from a frog was exposed to low concentrations of the "paralytic shellfish poison" the amplitude of

isotonic contractions decreased slowly both on direct stimulation of the muscle and on indirect stimulation through the nerve. Fig. 1 shows a tracing in which stimuli were applied at 1 min intervals to muscle and nerve alternately. Both nerve and muscle were immersed in modified Krebs solution bubbled with 5% carbon dioxide


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30 min    0.025   W 0.05    W      0.1   0.3                W
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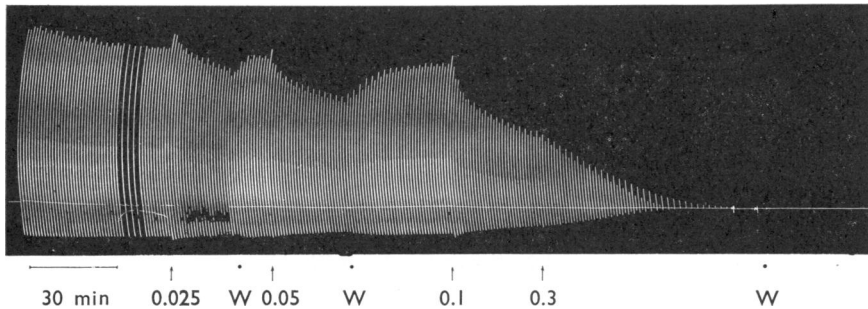
Fig. 1. Isotonic contractions of a frog gastrocnemius muscle. Direct stimulation (20 V, 0.5 msec duration) was alternated with indirect stimulation through the nerve (5 V, 0.1 msec duration), the latter being omitted for an 8 min period near the start. Both stimuli were supramaximal. The arrows mark the addition of " paralytic shellfish poison " to the organ-bath, at the concentrations indicated (μg/ml.). W indicates when the poison was washed out of the bath.

in oxygen. Indirect stimulation caused a contraction 2 to 3% smaller than that produced by direct stimulation. When the poison was added to the Krebs solution to make a concentration of 0.025 or 0.05 μg/ml. the contractions were slowly reduced in amplitude and there was scarcely any evidence of the indirect response being affected more than the direct response. At these low concentrations the effect came on slowly and, when the poison was removed with washes of fresh Krebs solution, both the contractions recovered. At higher concentrations of the poison (0.1 or 0.3 μg/ml.) the paralysis developed more rapidly, and if allowed to develop fully it could not be reversed by repeated washing. When the paralysis was almost complete there was a small differential effect, the indirect response being paralysed several minutes before the muscle ceased to respond to direct stimulation. There was no other indication that the contraction to indirect stimulation was paralysed more readily than to direct stimulation. This result contrasts with the clear differential block produced in similar preparations by the venom of the Indian cobra *Naja naja* (Fig. 2, *a*) or by curare (Fig. 2, *b*).

Recordings of the electrical waveforms from an endplate zone of a frog sartorius muscle were photographed while stimulating the nerve to the sartorius. Some of these are shown in Fig. 3 and it can be seen that the endplate potential and the muscle action potential were both diminished within a few minutes of adding the poison to the Krebs solution at a concentration of 0.025 μg/ml. Block of the muscle action potential did not unmask an endplate potential in the way that curare could. The muscle became paralysed to direct as well as to indirect stimulation ; when the poison was washed out with several changes of fresh Krebs solution the contractility and the action potentials slowly returned.
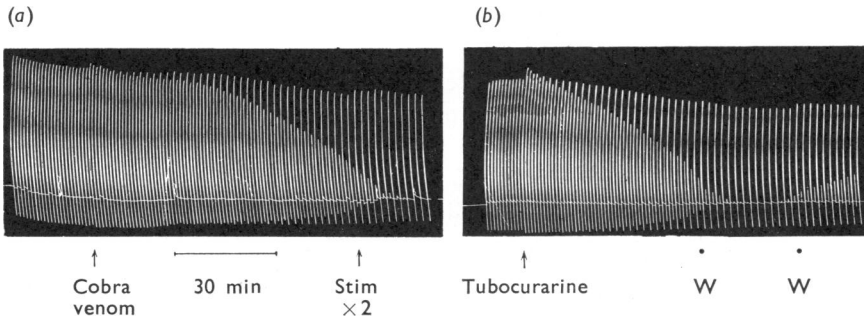
Fig. 2. Isotonic contractions of frog gastrocnemius muscles. Supramaximal (15 V, 0.5 msec duration) direct stimulation was alternated with supramaximal (1.25 V, 0.2 msec duration) indirect stimulation. In (a) *Naja naja* venom (10 μg/ml.) was added to the muscle-bath at the first arrow, and at the second arrow both stimuli were increased to double the initial voltage. In (b) D-tubocurarine (10 μg/ml.) was added to the muscle-bath, and later washed out at W.
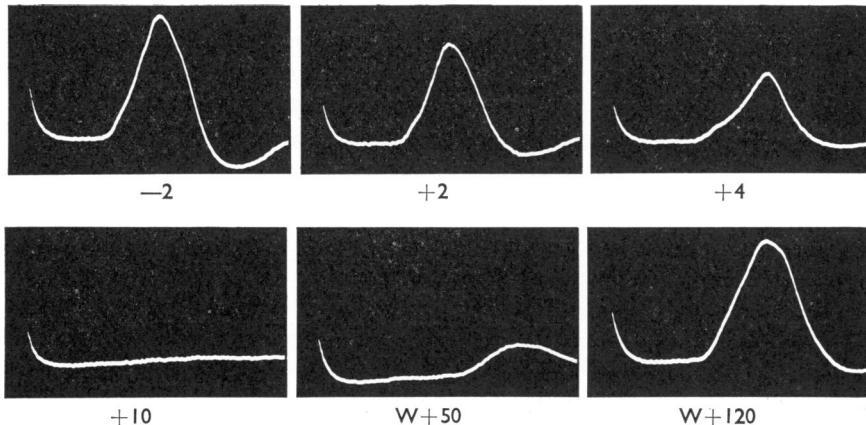

Fig. 3. Photographs of potentials displayed on an oscilloscope from the endplate zone of a frog sartorius muscle 2 min before, and 2, 4 and 10 min after adding " paralytic shellfish poison " (0.025 μg/ml.) to the muscle-bath, and 50 and 120 min after washing the poison out. The nerve to the sartorius was stimulated, the stimulus artifact starting at the left edge of each photograph. During the paralysis the recorded muscle action potential diminished steadily and there was no evidence of an endplate potential emerging as a distinct elevation on the upstroke of the action potential. Each photograph has a horizontal dimension of 5 msec and a vertical dimension of 2 mV.

Isolated nerve trunk preparation

If a substance is suspected of having a blocking action at the neuromuscular junction it is essential to establish that any experimentally observed differential paralysis cannot be due to block of nerve impulse conduction along the nerve trunks.

When recording the contractions of a nerve-muscle preparation, as illustrated in Figs. 1 and 2, it was usual to monitor the compound action potential from the nerve

before and after the preparation had been exposed to the poison. In many experiments the nerve action potential was not significantly changed after the muscle had been fully paralysed, but in a few experiments there was a definite reduction in the amplitude of the compound action potential after paralysing the muscle with the poison.

To investigate effects on nervous conduction with greater precision frog isolated sciatic nerves were set up in a trough with three chambers. The ends of the nerve lay in oil in the end chambers and electrodes allowed stimulation of one end and recording of the compound action potential from the other end. It was found that the shellfish poison could be added to the gassed Krebs solution in the middle chamber (through which 2.5 cm of the nerve trunk passed) without significantly blocking any of the elevations in the compound action potential. Concentrations of up to 0.5 μg/ml. of the poison had no significant effect even when left in the middle chamber for 1 hr, but did partly or wholly block conduction if left in overnight.

In the few nerve-muscle preparations that showed partial block of nerve conduction it was suspected that the poison might have penetrated the sheath at the cut spinal end of the sciatic nerve. Therefore pairs of frog sciatic nerves were set up side by side in the trough, one nerve being left intact while the second nerve had 2 to 3 mm of its sheath dissected away from the middle part of the trunk which was exposed to the poison. It was found that conduction in the desheathed nerve was rapidly blocked by concentrations of the poison that had no effect on the nerve whose sheath remained intact. Concentrations as low as 0.005 μg/ml. reduced the amplitude of the compound action potential in some desheathed nerves. Fig. 4 shows how 0.05 μg/ml. of the poison rapidly produced a partial block and an increase of latency in a desheathed nerve, the block becoming complete 4 min after adding the poison, whereas the nerve with an intact sheath was conducting with a normal amplitude and latency for a further 2 hr even though the concentration of poison had been raised to 0.45 μg/ml.

As well as the main group A elevation, conducted at 25 to 35 m/sec at room temperature, the responses of smaller diameter fibres were studied. For the sake of clarity these have not been shown on Fig. 4, but in one particular experiment an elevation conducted at about 3 m/sec was only partly blocked and was still present, but with a much increased latency, when the recording was terminated. However, in other experiments the small fibre response was blocked at least as quickly as the main group A response.

Injury potential of muscle

To determine whether the shellfish poison paralysed by depolarizing the nerve or muscle membrane, the injury potential was recorded from between the surface of a muscle and a crushed end. Sartorius muscles from frogs (in one instance a toad) were suspended in a moist atmosphere and the injury potentials were in the range 30 to 50 mV. Exposure of the muscles to concentrations of poison up to 5 μg/ml. produced paralysis but no significant change in potential. Exposure to 100 mM-potassium chloride solution very rapidly depolarized the muscles.
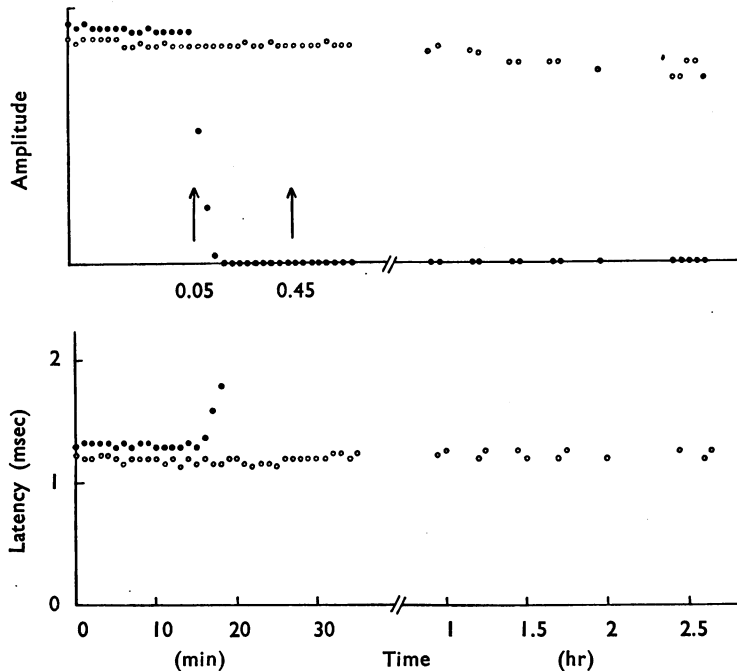
Fig. 4. Graphs showing the peak amplitudes (upper) and latencies (lower) of the main group A elevation in compound action potentials from a pair of frog sciatic nerves. O, nerve with intact sheath; ●, desheathed nerve. At the first arrow both nerve trunks were exposed to "paralytic shellfish poison," 0.05 μg/ml., which was increased to 0.45 μg/ml. at the second arrow. Note the change in time scale along the abscissa.

DISCUSSION

Kellaway (1935) came to the conclusion that the "paralytic shellfish poison" was a curarizing agent, and many later workers have subscribed to this view, although with some qualification. Kellaway based his conclusion on the observation that faradization of a frog muscle exposed to the poison in Ringer's solution could evoke a contraction at a time when indirect stimulation through the nerve had ceased to be effective. He does not seem to have tested whether the nerve was still conducting, nor does he comment upon the strength of the contraction at this stage. The work described here confirms that when both nerve and muscle are exposed to the poison the responses to indirect stimulation cease several minutes before those evoked by direct stimulation. However, this must be regarded as a pathological failure of transmission in a preparation in which the muscle itself is almost paralysed, for, as can be seen by comparing Figs. 1 and 2, there is no evidence of any appreciable degree of curarizing action when the total time-course of the paralysis is examined.

In rabbits Kellaway also thought that there was often a curarizing action at the phrenic nerve-diaphragm neuromuscular junction and sometimes at limb neuromuscular junctions. ·However, again he did not check the integrity of nervous conduction and I have unpublished observations which show that intravenous injec-

tion of the poison into rabbits can often block nervous conduction before completely paralysing limb muscles.

Any hypothesis based on neuromuscular junction block becomes untenable if low concentrations of poison directly block nervous conduction. The nerves of a normal frog sciatic nerve-gastrocnemius muscle preparation continued to conduct for a long time after exposure to quite high concentrations of the poison (0.1 to 0.5 μg/ml.) which completely paralysed the muscle, which result confirms Fingerman *et al.* (1953). A completely different situation exists, however, when the poison can gain access to the nerve fibres, either by immersion of the cut end in the solution, or more effectively and rapidly if the delicate perineurium is removed from a few millimetres of the trunk exposed to the poison (or if the poison is injected intravascularly in a mammalian preparation as mentioned above). Conduction along desheathed nerves was partly blocked by exposure to concentrations of poison as low as 0.005 to 0.01 μg/ml., which had little effect on muscular contraction, and was totally blocked in a few minutes by poison concentrations of 0.05 to 0.1 μg/ml., whereas the trunks of nerves with intact sheaths continued to conduct normally for at least 1 hr in solutions containing up to 0.5 μg/ml. of poison. This finding makes it difficult to interpret the results of Bolton *et al.* (1959), because it seems probable that the nerves which were stimulated to evoke the endplate potentials in their curarized muscles were wholly immersed in a solution containing 0.1 μg/ml. of poison. There is no evidence, therefore, of any specific block of the neuromuscular junction, because any differential paralysis of a nerve-muscle preparation can be accounted for by the block of nervous conduction with small concentrations of the poison.

The recordings from an endplate zone in sartorius muscle, reported in this paper, showed block of the muscle action potentials when both nerve and muscle were immersed in a solution containing 0.025 μg/ml. of poison. It is possible that even at this low concentration the poison was able to penetrate the cut end of the nerve sufficiently rapidly to affect nervous conduction; there was no sign that the muscle action potential diminished more rapidly than the endplate potential, so as to leave the latter in isolation, as happens when curare is used (Eccles, Katz & Kuffler, 1941).

It is surprising that the delicate perineurium that ensheaths the frog sciatic nerve should act as such an efficient barrier to the penetration of a simple substance with a molecular weight of 372 (Schantz, 1960), although this was recognized as a possibility by Fingerman *et al.* (1953).

The nitrogenous and strongly basic character of the poison (Schantz, 1960) made one suspect that it might act on nerve and muscle by depolarizing the cell membrane. However, the recording of injury potential in frog sartorius muscle showed no evidence of this action and further investigations are needed to find out how this poison is able to block conduction and to paralyse muscle. Tetrodotoxin, from globefish, has been reported to have similar "stabilizing" pharmacological effects upon frog nerve and muscle (Narahashi, Deguchi, Urakawa & Ohkubo, 1960). One would anticipate that a simple chemical substance that can block conduction in

such low concentrations without depolarization could be a valuable agent in fundamental physiological research.

REFERENCES

BOLTON, B. L., BERGNER, A. D., O'NEILL, J. J. & WAGLEY, P. F. (1959). Effect of a shell-fish poison on end-plate potentials. *Bull. Johns Hopk. Hosp.*, **105**, 233–238.

COVELL, W. P. & WHEDON, W. F. (1937). Effects of the paralytic shell-fish poison on nerve cells. *Arch. Path.*, **24**, 411–418.

D'AGUANNO, W. (1959). *Pharmacologist*, **1**, 71. Cited by Murtha (1960).

ECCLES, J. C., KATZ, B. & KUFFLER, S. W. (1941). Nature of the " endplate potential " in curarized muscle. *J. Neurophysiol.*, **4**, 362–387.

FINGERMAN, M., FORESTER, R. H. & STOVER, J. H. (1953). Action of shellfish poison on peripheral nerve and skeletal muscle. *Proc. Soc. exp. Biol. (N.Y.)*, **84**, 643–646.

KELLAWAY, C. H. (1935). The action of mussel poison on the nervous system. *Aust. J. exp. Biol. med. Sci.*, **13**, 79–94.

MEYER, K. F. (1953). Food poisoning. *New Engl. J. Med.*, **249**, 843–852.

MURTHA, E. F. (1960). Pharmacological study of poisons from shellfish and puffer fish. *Ann. N.Y. Acad. Sci.*, **90**, 820–836.

NARAHASHI, T., DEGUCHI, T., URAKAWA, N. & OHKUBO, Y. (1960). Stabilization and rectification of muscle fiber membrane by tetrodotoxin. *Amer. J. Physiol.*, **198**, 934–938.

PEPLER, W. J. & LOUBSER, E. (1960). Histochemical demonstration of the mode of action of the alkaloid in mussel poisoning. *Nature (Lond.)*, **188**, 860.

SAPEIKA, N. (1953). Actions of mussel poison. *Arch. int. Pharmacodyn.*, **93**, 135–142.

SCHANTZ, E. J. (1960). Biochemical studies on paralytic shellfish poisons. *Ann. N.Y. Acad. Sci.*, **90**, 843–855.

SCHANTZ, E. J. (1963). Studies on the paralytic poisons found in mussels and clams along the North American Pacific coast. In *Venomous and Poisonous Animals and Noxious Plants of the Pacific Region*, ed. KEEGAN, H. L. & MACFARLANE, W. V. London: Pergamon Press.

SCHANTZ, E. J., McFARREN, E. F., SCHAFER, M. L. & LEWIS, K. H. (1958). Purified shellfish poison for bioassay standardization. *J. Ass. off. Agric. Chem.*, **41**, 160–168.

SEVEN, M. J. (1958). Mussel poisoning. *Ann. intern. Med.*, **48**, 891–897.

SOMMER, H. & MEYER, K. F. (1937). Paralytic shell-fish poisoning. *Arch. Pathol.*, **24**, 560–598.

SOMMER, H., WHEDON, W. F., KOFOID, C. A. & STOHLER, R. (1937). Relation of paralytic shell-fish poison to certain plankton organisms of the genus Gonyaulax. *Arch. Path.*, **24**, 537–559.