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Two toxins from a poisonous sample of mussels *Mytilus edulis*

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Summary

1. A crude preparation of toxin was extracted from a sample of mussels *Mytilus edulis*, part of a batch responsible for many cases of paralytic shellfish poisoning.

2. The crude extract was partially purified by absorption on sodium Amberlite ion-exchange resin. Two toxins were recovered by elution from the Amberlite, and purified further by gel filtration.

3. One toxin closely resembled saxitoxin in its behaviour on Amberlite and in its biological effects.

4. The other toxin behaved quite differently on the Amberlite. Its molecule was small, comparable in size with saxitoxin. It was not tetrodotoxin. Its biological effects were similar, but not identical, to those of saxitoxin: it paralysed muscular contraction and inhibited conduction along nerves ; it caused death of experimental animals by producing a peripheral paralysis of respiration ; it did not depolarize the membrane of frog skeletal muscle fibres, but acted by preventing a stimulus from initiating a conducted action potential.

5. The biological effects of the second toxin suggest that, like saxitoxin and tetrodotoxin, it is an inhibitor of inward sodium ion movement through electrically excitable membranes.

Introduction

During May 1968 there was an outbreak of paralytic shellfish poisoning in Northumberland. About eighty people became ill after eating mussels (*Mytilus edulis*) which had been gathered near Holy Island, and large numbers of seabirds died along the North-East coast of Britain. This outbreak was associated with a bloom of the dinoflagellate *Gonyaulax tamarensis* in the offshore plankton (Wood, 1968), an organism that has previously been implicated in similar outbreaks in Canada (Needler, 1949 ; Prakash, 1963).

Paralytic shellfish poisoning has been studied in detail in California, where the mussels *M. californianus* became toxic after feeding on the dinoflagellate *G. catenella* (Sommer, Whedon, Kofoid & Stohler, 1937 ; Riegel, Stanger, Wikholm, Mold & Sommer, 1949). The poison is apparently identical to saxitoxin, the paralytic shellfish poison from the Alaskan clam *Saxidomus giganteus*, and the name saxitoxin is now commonly extended to include the poison from *M. californianus* and *G. catenella* (Mold, Bowden, Stanger, Maurer, Lynch, Wyler, Schantz & Riegel, 1957 ; Schantz, Mold, Howard, Bowden, Stanger, Lynch, Wintersteiner, Dutcher, Walters & Riegel,

1961 ; Schantz, Lynch, Vayvada, Matsumoto & Rapoport, 1966). The purification techniques developed by these workers enable saxitoxin to be obtained from extracts of toxic shellfish. However, these techniques have not been successful in purifying the poison of shellfish that have fed on *G. tamarensis* (Schantz, 1960).

The work described here was an attempt to extract saxitoxin from the Holy Island mussels which had caused the paralytic shellfish poisoning in May 1968. These shellfish were found to contain two neurotoxins having very similar actions. One, possibly identical to saxitoxin, was present in small amounts and another unknown toxin was present in greater quantities.

Methods

The starting material for the extraction of toxin was a sample of mussels, *Mytilus edulis* from a batch which had been gathered commercially from mussel beds near Holy Island, Northumberland, and which had caused an outbreak of paralytic shellfish poisoning in that area at the end of May 1968. These shellfish had been stored frozen from the time of the outbreak until extraction of toxins in December 1968. An acid extract of these mussels was processed by the techniques described by Schantz, Mold, Stanger, Shavel, Riel, Bowden, Lynch, Wyler, Riegel & Sommer (1957) using Amberlite ion exchange resin CG-50 (B.D.H., chromatographic grade type 1, 100–200 mesh). Minor variations from the procedures used by these workers are mentioned in the appropriate part of the **Results**.

Gel filtration of some of the fractions from the Amberlite columns was done on material evaporated to dryness *in vacuo*, redissolved in 0·003 M—0·05 M HCl and eluted with this solvent through columns of Sephadex G-10 or G-25 (Pharmacia Fine Chemicals).

The LD50 was determined for the crude extract and for the two toxic components by means of the method of Weil (1952) using mice weighing 18–25 g. The material was injected intraperitoneally in a weakly acid solution as recommended by Schantz, McFarren, Schafer & Lewis (1958). After the LD50 had been found, mean survival times were measured on groups of six–twelve mice given more than 1 LD50, and from these results a dose survival time curve was constructed as described in the **Results**. This curve was then used to assay chromatography column fractions for toxicity, using the methods described by Schantz *et al.* (1958) and Schantz (1960). The materials were usually injected in a volume of 0·5 ml. When good accuracy was required for determination of LD50 and dose survival times, the mice were weighed individually and the injected volume adjusted to keep the dose constant at 0·5 ml/20 g body weight.

The actions of the toxic fractions were compared with those of saxitoxin and tetrodotoxin. Saxitoxin was obtained through the generosity of Dr. E. J. Schantz (purified extracts of *Mytilus californianus* and *Saxidomus giganteus*). Tetrodotoxin was bought in the form of its citrate salt mixed with an excess of citric acid (Sankyo Co. Ltd.). The toxins were tested on the following preparations: desheathed frog sciatic nerve, desheathed *Taricha torosa* newt nerve, nerve filaments from rat cauda equina (Evans, 1968), frog sartorius nerve-muscle and the anaesthetized rabbit.

The isolated nerve preparations were set up in a three-chambered trough in which the ends of the nerve were in mineral oil for stimulation and recording, and the central portion of the nerve was irrigated with saline to which the toxins were added

after a control period. The saline for the amphibian tissues had the composition (mM): NaCl 110; KCl 2·5; $CaCl_2$ 1·8; glucose 5·6; Tris hydrochloride buffer (pH 7·4) 2–5. The Ringer-Locke for the rat nerve had the following composition (mM): NaCl 150; KCl 5·6; $CaCl_2$ 2·2; glucose 5·6; Tris hydrochloride buffer (pH 7·4) 2–5.

The salines were gassed with oxygen. In the experiments on the neuromuscular junction, glass micropipettes filled with 2·7 M KCl and having impedances in the range 7–20 MΩ, were used to record intracellular resting and end-plate potentials (e.p.p.). The steady resting potential was recorded on a potentiometric chart recorder (Bryans 27000) and the e.p.p. was A.C. coupled to an oscilloscope. All these experiments were carried out at room temperature (19–24° C).

The rabbits were anaesthetized with urethane 1·3–1·7 g/kg intravenously, sometimes supplemented with chloralose 25–30 mg/kg intravenously. Arterial blood pressure was measured through a Statham P 23 Db transducer connected by a polyethylene catheter to a femoral artery (1 mmHg≡1·333 mbar). Respiration was measured with the aid of a closed circuit spirometer in which the rise and fall of the bell rotated a low torque potentiometer to give an electrical analogue of tidal volume. Soda lime in the closed circuit absorbed CO_2; O_2 was injected at a rate sufficient to replace that used by the rabbit. The activity of the respiratory neurones in the C.N.S. was monitored by recording from the cut central end of one (or a portion of one) phrenic nerve. All these electrical signals were recorded on a multiple beam oscilloscope (Tektronix 565 with 3A3 and 3A74 pre-amplifiers) and on a polygraph (Devices M.4).

Further details of many of these procedures will be found in earlier publications (Evans, 1964, 1965, 1969a & 1969b).

Results

Administration of crude extract to mice

A preliminary investigation was made on an acid extract of poisonous mussels from Holy Island. This extract was generously provided by Mr. P. C. Wood (sample No. T.7) in October 1968. It was assayed on mice by intraperitoneal administration of serial dilutions, in a geometrical progression, chosen so that some dilutions gave less than 50% mortality within a group while other dilutions gave more than 50% mortality. The results were analysed with the aid of Weil's (1952) tables. It was found that this extract had an LD50 of $3{\cdot}925 \pm 0{\cdot}185$ µl when diluted with distilled water to 0·5 ml and injected intraperitoneally into a 20 g mouse. One LD50 is described as 1 mouse unit (M.U.), therefore this sample had a toxicity of 255 M.U./ml (see Schantz *et al.*, 1958).

Mice that died after administration of about 1 M.U. did so within 5–14 min after injection. When greater doses were given the mice died sooner and with less individual variation in the survival times. Mice were given stronger concentrations so that they received, in groups of six–twelve, doses between 1·02 and 4·45 M.U. in 0·5 ml/20 g body weight. The survival time of each mouse was measured from the commencement of intraperitoneal injection to the last breath. The mean time until death for each group, plotted against dose, gave a curve that was similar to that obtained with saxitoxin or tetrodotoxin.

Regression lines of the reciprocal of survival time, plotted against log dose, are approximately linear in the cases of saxitoxin (Schantz *et al.*, 1958) and tetrodotoxin, the slopes and intercepts being significantly different from each other (McFarren & Bartsch, 1960). Regression lines were therefore calculated for the Holy Island mussel extract, saxitoxin and tetrodotoxin after finding the LD50 of the two known toxins under identical conditions. Table 1 gives the values found for LD50, intercept and slope. Fig. 1 shows the regression lines flanked by confidence limits at ± 2 standard errors of the intercept. It can be seen that the poison present in the extract of Holy Island mussels appears to differ from saxitoxin and from tetrodotoxin, although the confidence limits overlap slightly.

TABLE 1. *Lethal doses (LD50) for 20g mice of saxitoxin, Holy Island mussel poison and tetrodotoxin*

	LD50	Intercept	Slope	Correl.	*n*
Saxitoxin	0·206±0·013 μg	0·1377±0·0085	0·4395±0·0238	0·9500	39
Mussel poison	3·925±0·185 μl	0·1104±0·0077	0·3873±0·0213	0·9446	42
Tetrodotoxin	0·26±0·02 μg	0·0557±0·0145	0·4121±0·0292	0·9420	27

The intercept ±S.E. at 1×LD50 and the slope ±S.E. of the regression lines of reciprocal of survival time in minutes against log dose in LD50 units, with correlation coefficients and numbers of mice used (*n*).

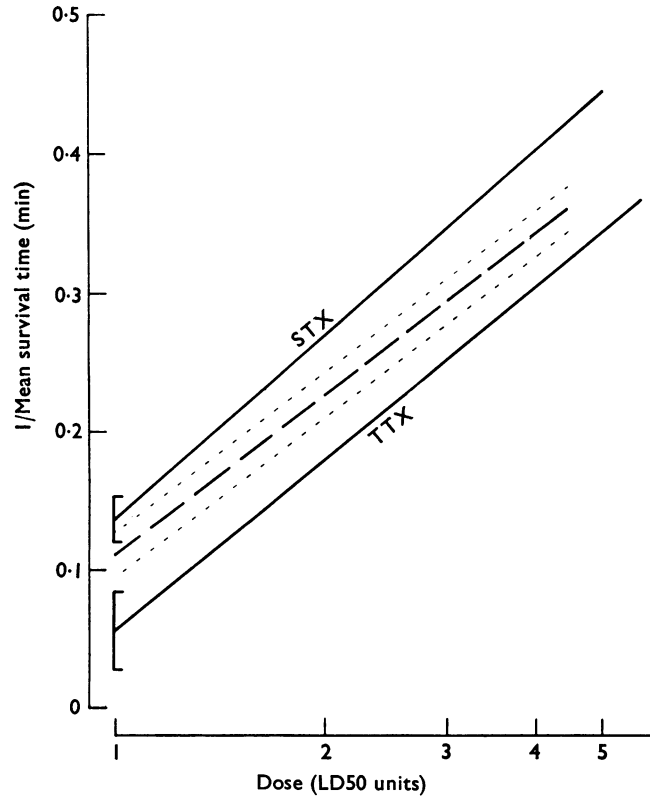

FIG. 1. Regression lines of the reciprocal of mean survival time in min (ordinate) plotted against dose of toxin in multiples of LD50 (abscissa, log scale). Upper solid line, saxitoxin (STX) with bracket to show ±2×S.E. of intercept. Bottom solid line tetrodotoxin (TTX) with bracket indicating ±2×S.E. of intercept. Centre dashed line, crude extract of poisonous mussels, with lightly dotted lines drawn parallel at ±2×S.E. of intercept to indicate confidence limits. All regression lines plotted from data in Table 1.

Purification of toxins from Mytilus edulis

Part of the first sample of extract (Mr. Wood's No. T.7) was passed through a small (1·0 × 15 cm) column of sodium Amberlite CG-50 and processed in the way described by Schantz *et al.* (1957) for the preparation of saxitoxin. It was found that most of the toxic material was eluted from the column during the preliminary wash with acetate buffer pH 4·0. Some saxitoxin-like material was bound to the resin and was later eluted with 0·5 M acetic acid. A few neuropharmacological tests, similar to those described below, were carried out on the toxic eluates. There was evidence that the toxin which had been eluted during the rinse with buffer pH 4·0 had different effects from that which remained bound to the Amberlite until it was eluted with acetic acid. A detailed investigation was then started with a larger quantity of raw material.

The starting material for the work to be described below was a 5 kg sample of mussels from Holy Island, part of the batch which had been responsible for the outbreak of a paralytic shellfish poisoning in May 1968. They had been kept frozen by the Ministry of Agriculture, Fisheries & Food at North Shields, Northumberland, and were in cold storage for about 6·5 months before extraction of toxins commenced. They were shucked, yielding 2·1 kg of soft tissue and shell liquor. This material was minced and macerated overnight at 4° C in 2 l. of 0·1 N HCl before homogenization in a blender. After a second overnight maceration at 4° C insoluble material was removed by centrifuging. The precipitate was re-extracted with 0·1 N HCl, centrifuged and the supernatant pooled with that from the first centrifugation. Ethanol, 15% by volume, was added as a preservative. The 4,760 ml of acid extract obtained was assayed for toxicity as described above and found to contain 18 M.U./ml, a total of 85,500 M.U. The toxicity was much lower than in Mr. Wood's T.7 sample, partly due to dilution when re-extracting the precipitate from the first centrifuging, but it may also indicate that there had been a loss of toxicity during the 6·5 months of cold storage.

About half the extract was adjusted to pH 5·4 with NaOH and passed onto a 4 × 88 cm column of sodium Amberlite CG-50, prepared as described by Schantz *et al.* (1957). The column was rinsed with distilled water and then eluted with acetate buffer pH 4·0. Schantz *et al.* (1957) used this procedure to remove inactive material from the column, leaving saxitoxin bound to resin. In the present experiments a lot of toxic material was eluted from the column by the buffer, the pH of the eluate being 5·4–4·5. After this toxin had been washed out of the column, 0·5 M acetic acid was used to elute more toxic material which appeared when the effluent pH was 3·5 The process was repeated with the second half of the extract, using a fresh column of Amberlite, with similar results. At this stage about 38,000 M.U. of toxin had been recovered in the acetate buffer and about 15,000 M.U. in the acetic acid. The remaining 32,500 M.U. of the material loaded onto the columns was unaccounted for; it was not recovered even when the columns were washed with 0·1 N HCl.

The toxic substance that bound weakly to the resin, so that 38,000 M.U. were eluted with acetate buffer, will be referred to as the major toxin throughout the rest of this paper. The other substance, which behaved like saxitoxin in that it bound more strongly to the sodium Amberlite, allowing 15,000 M.U. to be eluted with acetic acid, will be referred to as the minor toxin.

Both the major and the minor toxins were subjected to further purification procedures. These included repeated passage through smaller columns of sodium Amberlite, chromatography on hydrogen Amberlite CG-50 as described by Schantz *et al.* (1957) and gel filtration through 2×30 cm or 2×95 cm columns of Sephadex G.10 or G.25. In each case the effluent from the column was collected in 3, 7·5 or 10 ml fractions which were roughly assayed for toxicity. The most toxic fractions were pooled and evaporated *in vacuo* before being redissolved in a suitable solvent for the next stage of purification. Weakly toxic side-fractions were pooled and usually reworked.

The minor toxin was readily purified by chromatographic elution through a column of hydrogen Amberlite after preliminary fractionation on Sephadex. Losses were fairly high, and about 2,800 M.U. of this saxitoxin-like material was finally obtained in 1·8 mg total solids. This toxicity, of about 1,550 M.U./mg, compares satisfactorily with the values obtained by Schantz *et al.* (1957) for saxitoxin at this stage of purification. Because of the small amount of minor toxin now remaining it was decided not to proceed further to chromatography on alumina with which Schantz *et al.* (1957) raised the purity of their saxitoxin to 5,500 M.U./mg.

Attempts to purify the major toxin were much less satisfactory. No specific procedure was found which can be recommended. Due to its feeble affinity for Amberlite resin it was difficult to separate from inorganic salts, which were present in large amounts. When the major toxin was refractionated on sodium Amberlite, part of the toxic material was eluted with the initial distilled water rinse. In general, this eluted material closely resembled the major toxin (eluted later with acetate buffer) in its biological effects. However, occasional differences were noted, and it is possible that the material eluted in the water rinse was not identical to the major toxin. On 30 cm columns of Sephadex the major toxin was eluted in virtually the same fractions as the salt, indicating a low molecular weight. On a 95 cm column of G.10 Sephadex, using 3 mM HCl as solvent, the toxin was eluted slightly later than the NaCl, indicating a tendency to be adsorbed onto the gel. This permitted a partial separation from salts, and by repeating the process the toxicity was raised to 270 M.U./mg. By means of flame photometry and chloride estimation it was found that approximately 90% of the remaining solid was NaCl, therefore this material was pharmacologically satisfactory for further biological testing. The losses at each stage were high even when the side-fractions were reworked. Further attempts to purify this toxin were halted when only 500 of the original 38,000 M.U. remained.

Dose survival times in mice

After determining the LD50 of the major and minor toxins, a dose survival time regression line was found for each toxin, using doses up to 6·4 × LD50. These regression lines were similar to Fig. 1 and there were no significant differences either between the major and minor toxins or between them and the crude extract, T.7, the regression line of which is shown in Fig. 1. The regression line of the minor toxin lay closer to the saxitoxin line than the major toxin did. The confidence limits of the saxitoxin and minor toxin regression lines overlapped to some extent, whereas the regression line of the major toxin appeared to be significantly different from saxitoxin, though not from tetrodotoxin.

No differences were noted between the major and minor toxins when observing the behaviour of the mice that had received lethal doses of these fractions. The behavioural effects of the two toxins were essentially similar to the effects of a lethal dose of saxitoxin.

Stability of the toxins when heated in acid solution

The major and minor toxins were each diluted to a concentration of 10 M.U./ml, either with NaH_2PO_4 10 mM or with 0·1 N HCl. These diluents gave a final pH of about 4·5 and 1, respectively. The ability of the toxins to withstand heating at these pH values was tested by placing test tubes of these dilutions in a boiling water bath for times ranging from 5–40 min. The tubes were then cooled quickly and 0·5 ml was injected intraperitoneally into mice. In the case of the dilutions made with 0·1 N HCl, excess acidity was neutralized by adding a few drops of 5N NaOH to bring the pH into the range 2–5 before injecting. Remaining toxicity was estimated from the dose survival time regression lines.

There was not much loss of toxicity on heating any of the toxin dilutions; there was no apparent difference between the major and minor toxins, and the toxins seemed to be as stable at pH 1 as at pH 4·5. After heating for 30 or 40 min at 100 °C less than half the toxicity was destroyed. For comparison, saxitoxin was treated in the same way, diluted to a comparable concentration (2 μg/ml). Its behaviour on heating was indistinguishable from the Holy Island mussel poisons.

Tetrodotoxin was also heated at pH 1 and at pH 4.8. At the latter pH it was fairly stable, losing about half the toxicity after 30 min, but at pH 1 it was more rapidly destroyed, about half the toxicity being lost in 5–10 min at 100° C. The relative instability of tetrodotoxin at pH 1 clearly distinguishes it from saxitoxin and from the Holy Island mussel poisons.

Effects on nerve conduction

The major and minor toxins were tested *in vitro* for their effects on the conduction of nerve impulses in the desheathed frog sciatic nerve, the desheathed newt sciatic nerve and in filaments from the rat cauda equina. When tested on frog nerves, both the toxins prevented conduction when they were added to the bath saline in concentrations greater than 50 M.U./litre. At concentrations of 100–200 M.U./l. both toxins produced, within 5–15 min, a diminution in amplitude of the compound action potential similar in degree to that produced by an equivalent concentration of saxitoxin (20–40 μg/litre).

Fig. 2 illustrates an experiment on a frog sciatic nerve exposed to saxitoxin 20 μg/l., the major toxin 100 M.U./l. and the minor toxin 100 M.U./litre. The nerve was washed for one hour in between each exposure to toxin. Each application of toxin caused the compound action potential to diminish and the three toxins are comparable in their potencies. The effects are readily reversed by washing, the rate of recovery being similar in all cases.

Lower concentrations of the toxins depressed conduction in the rat cauda equina nerves. A concentration of 25 M.U./l. reduced the amplitude of the compound action potential to 50% within 6–8 min exposure; 55 M.U./l. blocked conduction completely in about 10 minutes.

The toxins were tested on desheathed sciatic nerves from the newts *Triturus cristatus* and *Taricha torosa*. In the latter species especially there is a marked difference between the blocking actions of saxitoxin and tetrodotoxin (Kao & Fuhrman, 1967), nerve conduction being very resistant to tetrodotoxin. The major and minor toxins both blocked conduction when applied to the desheathed segment (Fig. 3a & b) in concentrations equivalent, in terms of M.U./l., to a concentration of saxitoxin which caused a comparable block (Fig. 3c). The same concentration of tetrodotoxin had no significant effect (Fig. 3d).

In all these experiments the degree of block of the conducted compound action potential produced by the minor toxin closely matched that produced by an equivalent concentration of saxitoxin (0·206 μg=1 M.U.). In several experiments the major toxin seemed to be slightly less potent than saxitoxin (in terms of M.U./l.) in its ability to block nerve conduction. This slight difference can be seen in Fig. 2. However, the variation present in these experiments makes it impossible to give a meaningful quantitative comparison of potencies on nerve conduction, the differences being small in all cases.

Effects on muscle contraction

When they were applied to the sartorius nerve-muscle preparation of a frog (*Rana temporaria* or *R. pipiens*) both of the toxic components of the Holy Island mussel poison abolished the contractions. The contractions elicited by direct stimulation of the muscle were abolished as well as those elicited indirectly by stimulation of the motor nerve. The toxins were added to the bath saline to give concentrations rang-

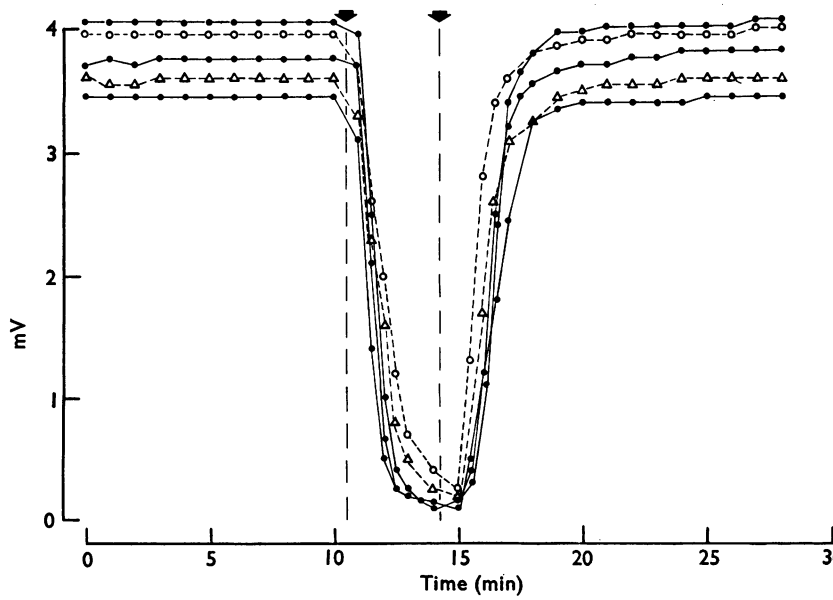

FIG. 2. Peak amplitude of the compound action potential conducted in frog sciatic nerve (ordinate in mV) plotted against time (min). During the time between the arrows the central (desheathed) part of the nerve was exposed to STX 20 μg/l. (●), major toxin 100 M.U./l. (○), and minor toxin 100 M.U./l. (△). The curves were obtained at 1 h intervals, to allow full recovery during the period of washing between the tests.

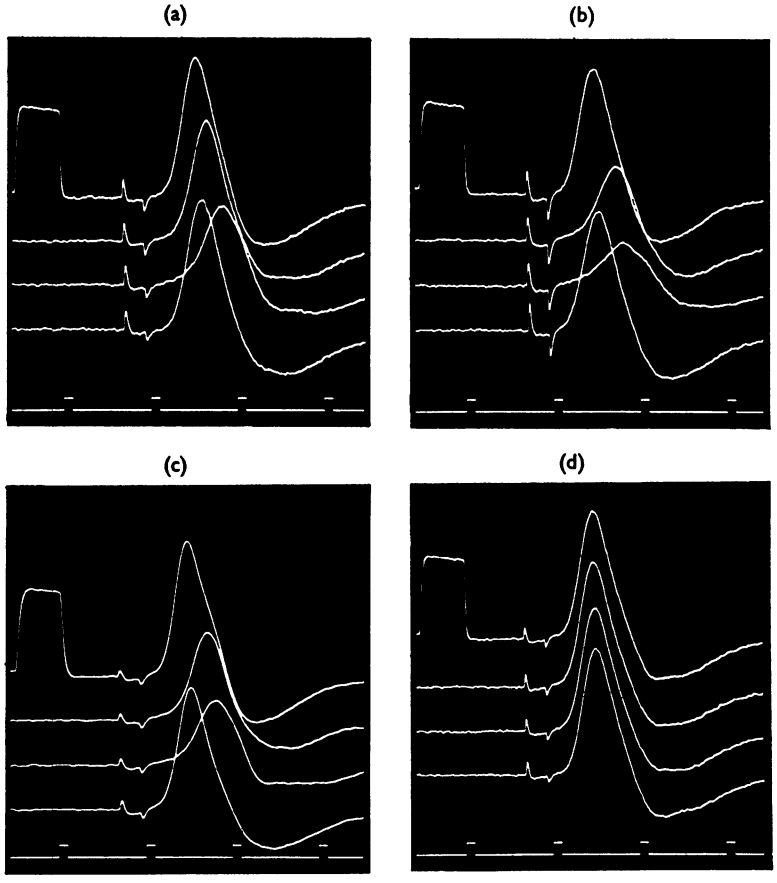

FIG. 3. Compound action potentials recorded from sciatic nerve of newt *Taricha torosa*. Each photograph carries the following traces, read from above downwards: (i) 1 mV calibrating pulse followed by control response ; (ii) response after 3 min exposure to toxin ; (iii) response after 9 min exposure ; (iv) response recovered after 11–12 min washing ; (v) 1 ms timing pips. The nerve was exposed to (a) the major toxin 9 M.U./ml ; (b) minor toxin 10 M.U./ml ; (c) saxitoxin 2 μg/ml ; (d) tetrodotoxin 2 μg/ml.

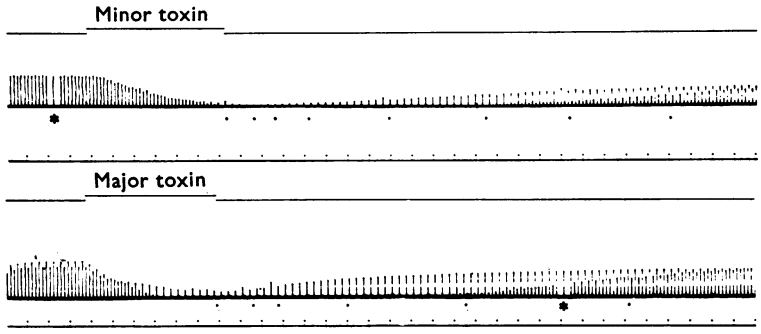

FIG. 4. Two traces of the twitch contractions of a frog sartorius muscle stimulated every 10 s. The stimuli were given alternately to the muscle (direct) and to the nerve (indirect). In each trace two indirect stimuli have been omitted at the asterisk*. The signal mark above each trace indicates exposure to minor toxin 100 M.U./l. (upper trace) or to major toxin 100 M.U./l. (lower trace). The toxins were washed out by repeatedly changing the bath fluid at the times marked (●). The same preparation was used for both traces, 1·75 h being allowed for recovery between the tests. Time marks, 1 min intervals.

ing from 50–167 M.U./litre. The effects of comparable concentrations of saxitoxin and tetrodotoxin were also examined.

Fig. 4 shows two tracings taken from one of these experiments. Each trace shows a series of muscle twitches, direct and indirect responses alternating at intervals of 10 s. In the upper trace, the signal above the responses marks the application of the minor toxin at a concentration of 100 M.U./l. The direct and indirect responses both diminished at the same rate and the muscle was almost completely paralysed in 6·5 min. When the toxin was washed out by repeatedly changing the bath saline (changes are marked by dots below the trace) both responses recovered, the indirect response taking longer to recover than the direct response. When saxitoxin was later added in a comparable concentration, in terms of M.U./l., an identical effect was obtained.

The lower trace in Fig. 4 shows that the major toxin also paralysed the contractions, with some small points of difference. The indirect response was lost sooner. When the toxin was washed out, the difference between the rates at which the direct and indirect responses recovered was even greater than the difference seen with the minor toxin. This tendency for the major toxin to affect the indirect response proportionately more than the direct response was seen in several of these experiments. Although the differences between the major and minor toxins were slight in this respect they are thought to be significant, especially in view of the effects seen at individual neuromuscular junctions (see below).

Effects on resting muscle membrane potential

Penetration of frog muscle fibres by KCl-filled glass micropipettes allowed the intracellular resting potential to be measured. In most fibres of the sartorius muscle

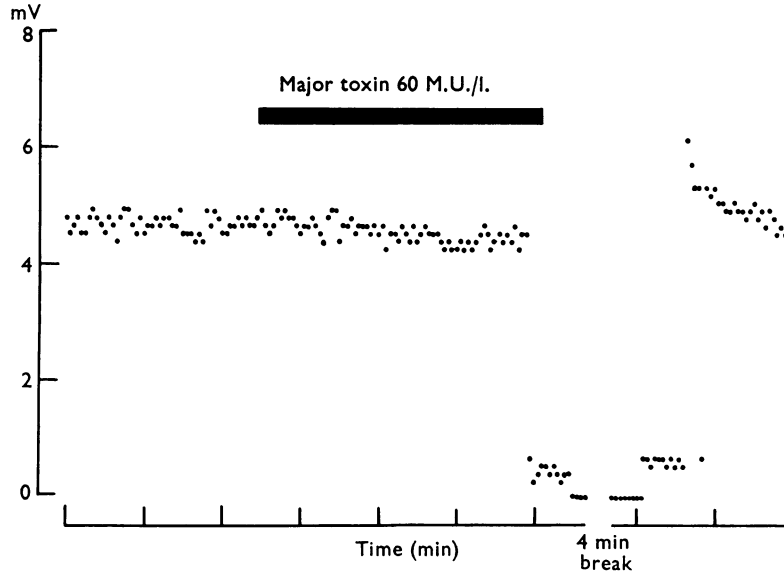

FIG. 5. Peak amplitudes of individual end-plate potentials in a frog sartorius muscle (ordinate in mV) plotted against time (min) with a 4 min break where indicated. The bar marks the addition of major toxin 60 M.U./l. to the perfusing saline. d-Tubocurarine 2·5 mg/l. was present in the saline throughout the experiment. Responses were evoked every 3 seconds.

this potential was in the range −80 to −95 mV. The limit of accuracy with which the potential could be measured over a period of 10–30 min was about ±2 mV, because of slight drifting of amplifier and electrode potentials occurring during this period. Within these limits of accuracy, it was found that neither of the toxins from the Holy Island mussel poison had any effect on resting muscle membrane potential.

Effects on end-plate potentials

When a micropipette penetrated a frog muscle fibre near a neuromuscular junction, the localized end-plate potential (e.p.p.) could be recorded after the arrival of an impulse at the motor nerve terminal. In these experiments the saline perfusing the muscle bath contained sufficient curare or $MgCl_2$ to prevent nerve stimulation from causing a muscle twitch. Under these circumstances the e.p.p. was recorded as a localized depolarization with an amplitude of a few mV. The actions of the two toxins from the Holy Island mussel poison were tested by adding these toxins for a short time to the perfusion saline. The concentration in the saline was usually 50–80 M.U./litre.

The typical effect of the major toxin was to increase the latency of the e.p.p with little if any initial decrease in amplitude. The latency increased over a period of

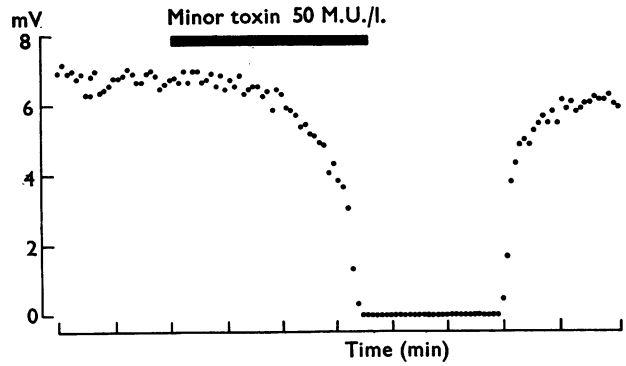

FIG. 6. Peak amplitudes of individual end-plate potentials in a frog sartorius muscle (ordinate in mV) plotted against time (min). The bar marks the addition of minor toxin 50 M.U./l. to the perfusing saline, which contained d-tubocurarine 2 mg/l. throughout the experiment. Responses were evoked every 5 seconds.

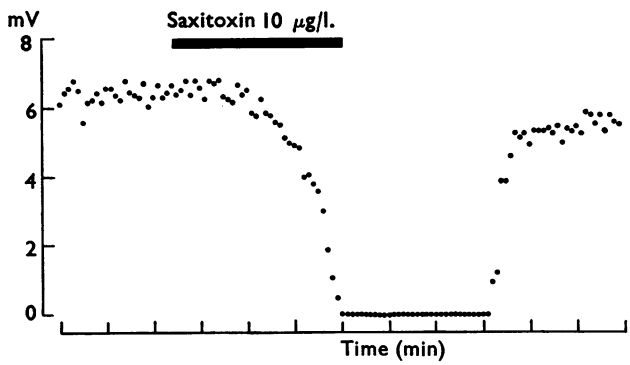

FIG. 7. Same end-plate as Fig. 5, recorded 1 h later. The bar marks the addition of STX 10 μg/l. to the perfusing saline.

several minutes, eventually reaching a value approximately 60% greater than the control. At this point the e.p.p disappeared abruptly, indicating that conduction along the motor nerve had become blocked. In a few experiments the disappearance of the e.p.p. took place in two or three well defined steps, the amplitude falling sharply to a lower level, where it remained for a short time before abruptly falling further.

Fig. 5 is a graph from one such experiment, showing the e.p.p. amplitude falling suddenly from a mean of 4·4 mV to about 0·5 mV after exposure to the major toxin 60 M.U./l. for 3·5 min. Thirty seconds later the remaining e.p.p disappeared abruptly. In these experiments the toxin was washed out as soon as the e.p.p. had disappeared or fallen to a low amplitude and the responses then reappeared after a period of washing that usually lasted for several minutes. In the experiment shown in Fig. 5 recovery took more than 5 min and, like the disappearance, the e.p.p recovered in two abrupt steps. In very few experiments did the amplitude fall considerably before disappearing abruptly. In all the experiments with the major toxin it was usual for both the amplitude and the latency of the e.p.p to be greater than the control value for a short time after the abrupt recovery.

The minor toxin had a different effect at the frog neuromuscular junction. The typical effect of this toxin was to reduce the amplitude of the e.p.p gradually, after a short latent period. It generally took 1–5 min for the minor toxin to reduce the e.p.p to zero and during this time it was unusual to observe any sudden fall in amplitude. The latency of the e.p.p increased as the amplitude fell, but to a smaller extent than that seen in the experiments with the major toxin. When the toxin was washed out as soon as the e.p.p had fallen to zero, the response usually recovered smoothly. A small e.p.p. appeared within a minute or so of washing and increased steadily in amplitude during the next few minutes. Occasionally, however, the e.p.p reappeared abruptly, almost reaching the control amplitude. Abrupt recoveries were unusual in the experiments with the minor toxin and were generally seen only when there had been a delay before starting to wash out the toxin after the e.p.p had diminished to zero.

Fig. 6 is a graph of e.p.p. amplitudes taken from an experiment in which the frog sartorius muscle was exposed for 3·5 min to the minor toxin 50 M.U./l. The e.p.p amplitude slowly fell to zero. After about 2·5 min washing the responses began to recover, increasing steadily towards the control amplitude. After washing for 1 hour the same preparation was then exposed to saxitoxin 10 μg/litre. The results of this test are shown in Fig. 7 and it can be seen that the minor toxin 50 M.U./l. and saxitoxin 10 μg/l. were indistinguishable in their effects.

Effects on the blood pressure and respiration of rabbits

The toxins were diluted with Ringer-Locke to a concentration of 10 M.U./ml and this was administered by slow intravenous infusion at a constant rate. Both the major and the minor toxins produced similar effects on respiration and blood pressure as shown in Figs. 8 & 9. After a small amount of toxin had been infused (the dose varying in different animals from 3–7 M.U./kg) the arterial blood pressure began to fall. It fell further, with little change in heart rate, with further infusion of toxin. The mean blood pressure often fell to as low as 40 mm Hg after large amounts of toxin, but the heart continued to beat for a few minutes after respiratory movements had ceased.

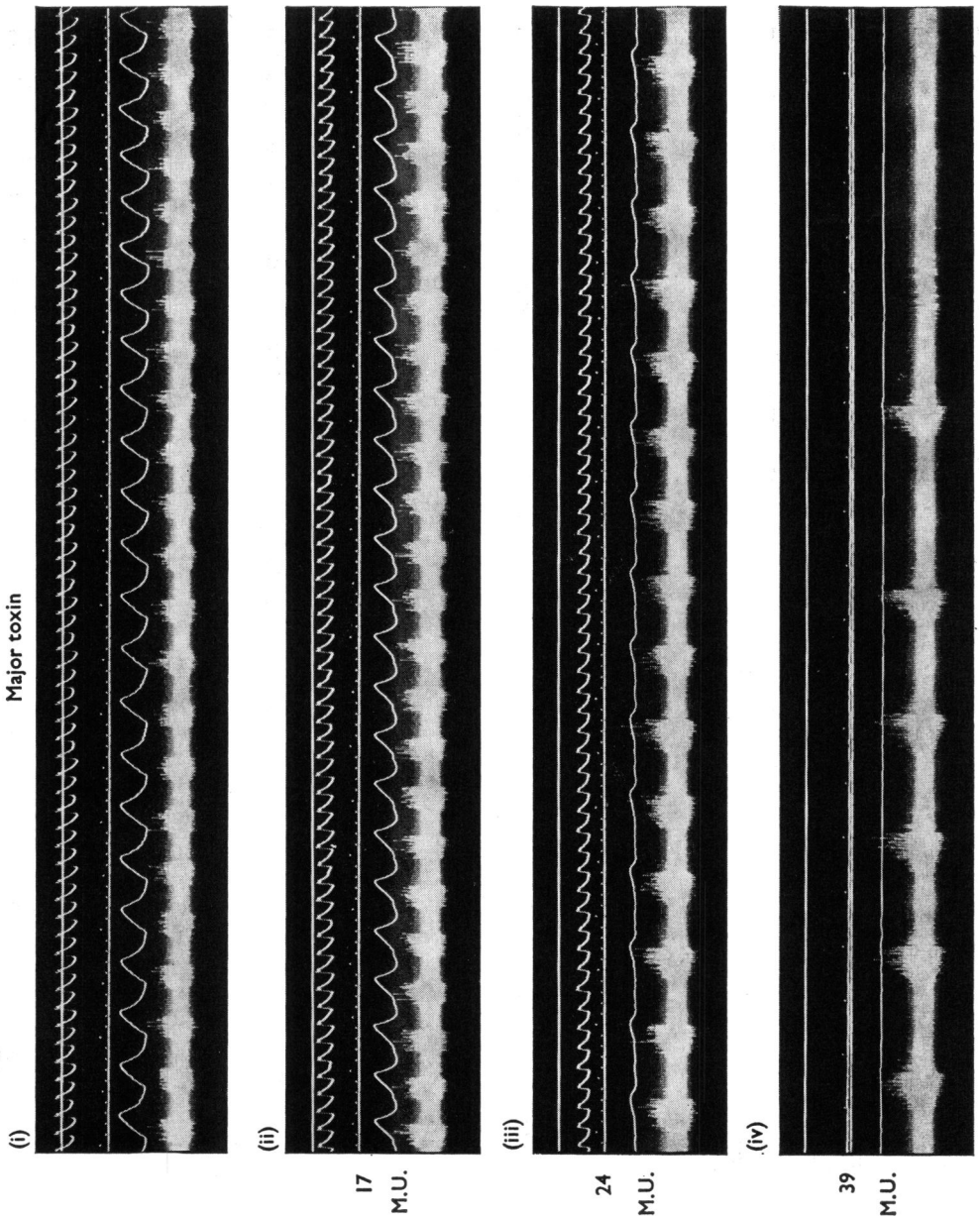

FIG. 8. Records from a 3·5 kg rabbit anaesthetized with urethane and chloralose. The traces in each strip are, from above downwards: 100 mmHg pressure level; arterial B.P.; 0 mmHg pressure level with timing pips at 0·1, 0·5 and 1·0 s intervals; respiratory movements (insp. ↑); electroneurogram recorded from the central cut end of the right phrenic nerve. (i), Control traces taken 20 s before starting to infuse major toxin intravenously at rate of 1 M.U. in 21 s; (ii), 357–367 s after start of infusion, 17 M.U. given; (iii), 514–524 s after start, 24 M.U. given; (iv), 820–830 s after start, 39 M.U. given. Respiratory movements had ceased about 4 min earlier and the ventricles stopped beating a few seconds before the record was taken, which shows the terminal barrages of impulses in the phrenic nerve.

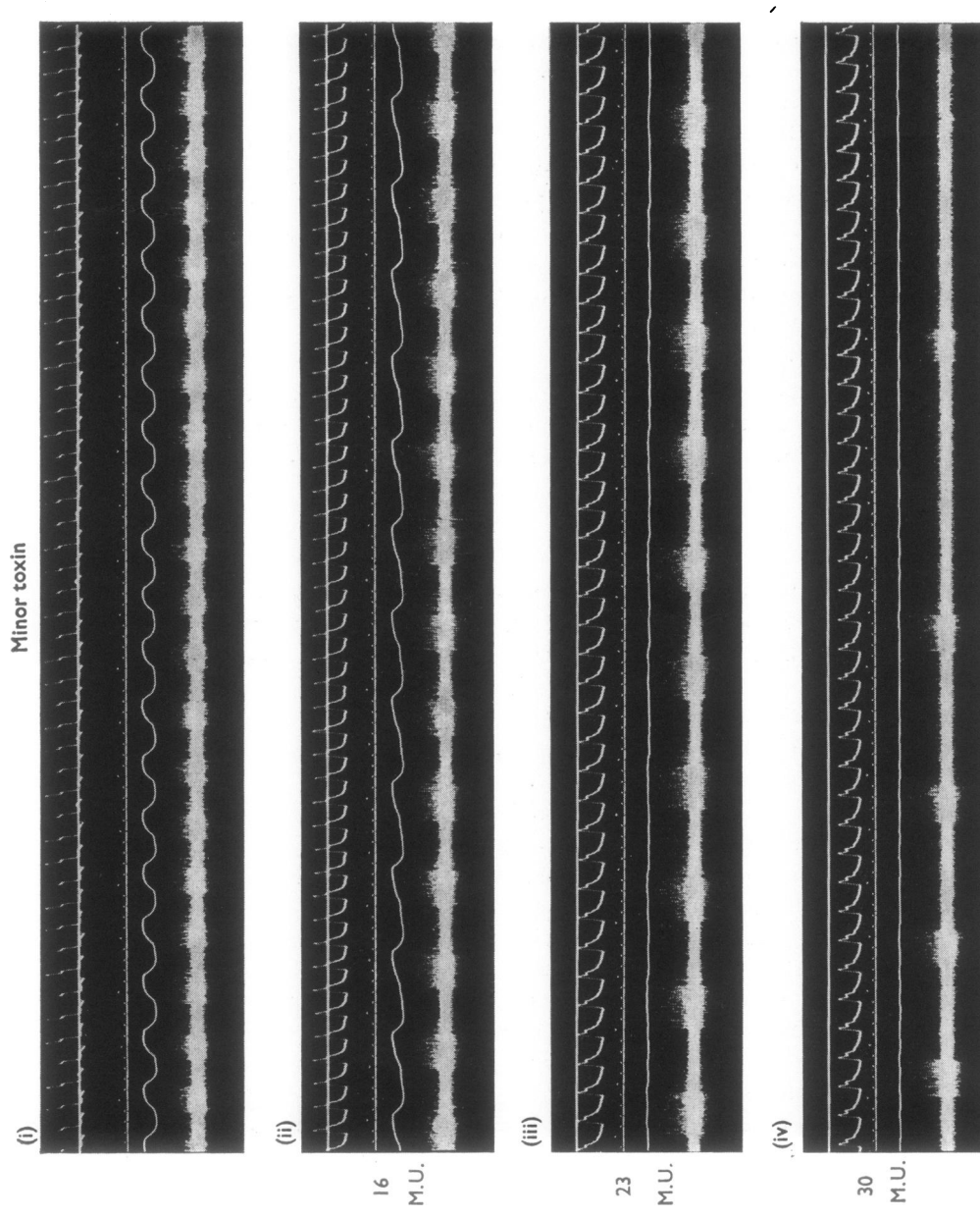

FIG. 9. Records from a 2·75 kg rabbit anaesthetized with urethane. Minor toxin was infused intravenously at constant rate of 1 M.U. in 23 s. The traces show the same parameters as in Fig. 7. (i), Control traces 30 s before starting to give toxin ; (ii), 361–371 s after start, 16 M.U. given ; (iii), 528–538 s after start, 23 M.U. given ; (iv), 682–692 s after start, 30 M.U. given. This strip shows the terminal barrages of impulses in the central cut end of a part of the right phrenic nerve. Respiratory movements had ceased 1·5 min previously.

Changes in the respiratory movements began to appear at about the same dose at which the blood pressure began to fall. At first the only change was a slight slowing of respiratory rate associated with a longer inspiratory phase. At this stage there was usually some increase in the motor nerve discharge recorded from the cut central end of one phrenic nerve (Figs. 8 (ii) & 9 (ii)). As infusion of toxin continued the respiratory movements became shallow, but the barrages of impulses in the phrenic nerve increased (Figs. 8 (iii) and 9 (iii)). By the time that about 10 M.U./kg had been infused the respiratory movements had virtually ceased, but massive bursts of motor impulses continued in the phrenic nerve as the medullary respiratory centre responded to the asphyxial drive. Eventually asphyxia overcame the C.N.S. and the phrenic nerve barrages slowed and then stopped (Fig. 8 (iv) and 9 (iv)). In some animals the ventricles stopped beating at about this time (Figs. 8 (iv)). But usually the heart continued to beat for a minute or two after the phrenic nerve had fallen silent (Fig. 9 (iv)).

These effects produced by the major and minor toxins are indistinguishable from those seen when saxitoxin is infused slowly into anaesthetized cats or rabbits (Evans, 1965) and are also similar to those seen when tetrodotoxin is given to rats (Sakai, Sato & Uraguchi, 1961 ; Cheng, Ling & Wang, 1968).

Discussion

Outbreaks of paralytic shellfish poisoning have been common occurrences in some parts of the world (see Halstead, 1965 ; Kao, 1966) and the action of the poison on human victims is now well documented (e.g. Seven, 1958). Few outbreaks have occurred in Europe, so it was of considerable interest when there was an outbreak in Northumberland in May 1968. The neurological effects produced by this outbreak seemed similar to those described by Seven (1958) and others, although there were some minor points of difference (H. A. Dewar & R. C. M. Pearson, personal communications, June 1968). When it was found that the development of toxicity in the mussels at Holy Island had been preceded by a bloom of the dinoflagellate *Gonyaulax tamarensis* in the North Sea plankton, it seemed possible that the outbreak of mussel poisoning in Northumberland might have been caused by a substance different from saxitoxin, which is responsible for the classical Californian paralytic shellfish poisoning. The reasons for suspecting a difference were as follows: In North America mussels (*Mytilus californianus*) derive their toxicity from a different species of dinoflagellate, *G. catenella* (Sommer, *et al.*, 1937). The poison from these mussels has been purified and is almost certainly identical to saxitoxin, the paralytic shellfish poison from the Alaskan butterclam *Saxidomus giganteus* (Schantz *et al.*, 1961 ; Schantz, 1967). The dinoflagellate *G. tamarensis* has not been implicated in Californian outbreaks of paralytic shellfish poisoning, but it does seem to have been associated with an outbreak of poisoning in the Bay of Fundy, on the Atlantic coast of Canada (Needler, 1949 ; Prakash, 1963). Attempts to use standard saxitoxin purification procedures to extract the poison from Bay of Fundy scallops failed (Schantz, 1960 and personal communication, 1968) and therefore it seemed likely that *G. tamarensis* produced a type of paralytic shellfish poison that was different from saxitoxin.

The results of the experiments described here show that the mussels, *M. edulis*, which had been feeding near Holy Island when *G. tamarensis* was prevalent in the plankton, acquired a powerful neurotoxin different from saxitoxin. At the same

time, smaller amounts of a second toxin, closely resembling saxitoxin, have been extracted from these shellfish.

Fractionation of an acid extract from toxic Holy Island mussels has yielded two components. The component responsible for most of the toxicity (the major toxin) did not bind well to the sodium Amberlite, while the other toxin (the minor toxin) behaved like saxitoxin and needed acetic acid to elute it from the resin. The minor toxin could be concentrated by using standard saxitoxin purification procedures, and was worked up to a potency of 1550 M.U./mg without difficulty. Using the same procedures Schantz *et al.* (1957) purified saxitoxin to 2500 M.U./mg, and by further chromatography on alumina they eventually raised the purity of saxitoxin to 5500 M.U./mg. These stages of chromatography were not attempted in the present work because of the small quantity of material remaining after partial purification.

The present experiments were not so successful in purifying the major toxin, even the best fractions of which contained excess of inorganic salts, and the potency was only 270 M.U./mg. Nevertheless the organic content of this material was low, the main contaminant being sodium chloride. The results of pharmacological tests with this material seemed to be free from interference, and it is therefore assumed that they are a reliable indication of the actions of the major toxin.

The two toxins resembled each other in many of their effects. When they were injected into mice they could not be distinguished, either by the pattern of their effects or from the dose survival time relation. The calculated regression lines for this relation differed slightly, the line of the minor toxin being closer to that of saxitoxin, but the differences were not statistically significant.

Both the major and minor toxins stopped conduction of nerve impulses in desheathed frog nerve and in filaments from the cauda equina of the rat. Both paralysed the direct and indirect twitch contractions of frog muscle, but did not affect the resting muscle membrane potential. No qualitative differences were seen between their effects on these preparations. The effects were produced by equivalent concentrations of the two toxins, measured in M.U./l., with a slight difference sometimes noted between their relative potencies. On these preparations saxitoxin and tetrodotoxin both produce effects similar to the effects of the Holy Island mussel toxins and at similar concentrations.

The two toxins blocked conduction of action potentials when applied to the desheathed portion of nerve from the Californian newt *Taricha torosa*. Rather high concentrations, 5–20 M.U./ml, were needed to achieve such a block but saxitoxin produced a similar degree of block when applied at equivalent concentrations. This preparation clearly distinguished these substances from tetrodotoxin, which has no effect at comparable concentrations (Kao & Fuhrman, 1967).

When given intravenously to anaesthetized rabbits both toxins produced hypotension and respiratory arrest. The respiratory paralysis was found to be due to a direct action of the toxins on the respiratory muscles or nerve terminals; the medullary centres were not depressed after intravenous administration. In this respect also, the toxins resemble saxitoxin and tetrodotoxin (Sakai, Sato & Uraguchi, 1961; Evans, 1965; Cheng *et al.*, 1968).

Qualitative differences between the effects of the two toxins were seen consistently in one preparation, the neuromuscular junction of the frog sartorius muscle. Saxitoxin and tetrodotoxin also show differences between their actions on this prepara-

tion (Evans 1969b). At the frog's neuromuscular junction the minor toxin caused a progressive graded reduction in the amplitude of the end-plate potential (e.p.p.). This effect was reversible by washing and the recovery of the e.p.p was progressive in most cases. In this, as in all its other properties, the minor toxin resembled saxitoxin (Nishyama & Kao, 1964). When the major toxin was tested on this preparation it caused an abrupt disappearance of the e.p.p after a latent period, during which conduction along the motor nerve slowed. The effect was reversible but rather prolonged washing was needed before the e.p.p re-appeared as suddenly as it had disappeared. These effects suggested that the major toxin was acting to block conduction along the motor nerves without affecting transmission at the neuromuscular junction. These effects are similar to those produced by tetrodotoxin (Furukawa, Sasaoka & Hosoya, 1959).

Although there were many similarities between the effects of tetrodotoxin and the major toxin, the experiments on *Taricha torosa* nerve showed that they are not identical. This difference was confirmed by finding that the major toxin of the Holy Island mussels was heat stable at pH 1 and at pH 4·5. Tetrodotoxin, on the other hand, was unstable at pH 1, although it was reasonably stable at pH 4·8.

Nothing is known about the chemical structure of the major toxin, except that it is a small molecule. It is unlikely to be a postmortem breakdown product of the minor toxin or saxitoxin, because the ratio of major/minor toxicities was similar in the mussels extracted after 6·5 months' storage, to the ratio in the extract (T.7) prepared by Mr. Wood soon after the clinical outbreak. Saxitoxin is reasonably stable, 80–100% of the toxicity of *M. californianus* and *S. giganteus* being recoverable as saxitoxin when extracts of these shellfish are fractionated (Schantz *et al.* 1957) and there do not seem to be other toxins present in appreciable quantities. Saxitoxin derivatives, prepared in the laboratory, are non-toxic (Schantz, 1960). It would seem likely that the major and minor toxins are chemically related and that they are perhaps produced by similar metabolic pathways in the dinoflagellate *G. tamarensis*. It seems that saxitoxin is the only poisonous metabolite produced by *G. catenella*, and as the minor toxin has been indistinguishable from saxitoxin, it is reasonable to assume that the major toxin may be chemically related to saxitoxin. One cannot yet say whether it may be related to saxitoxin-like materials that have been extracted from other organisms (Casselman, Greenhalgh, Brownell & Bannard, 1960 ; Jackim & Gentile, 1968).

The general effects of the major toxin show that it is not a depolarizing agent, but that it prevents activity in electrically excitable membranes. It therefore appears to be an inhibitor of inward sodium ion movement through these membranes, and hence falls into the same category of agents as tetrodotoxin and saxitoxin. If it could be obtained and purified on a larger scale it might be a useful addition to these two toxins for use in research into membrane physiology. The results of the experiments on the neuromuscular junction suggest that it differs from saxitoxin in its permeability properties. If it is found to penetrate the blood-brain barrier better than saxitoxin and tetrodotoxin it would be useful as a tool in C.N.S. research. It has been suggested (H. A. Dewar, personal communication) that patients in the May 1968 outbreak did show some symptoms indicative of C.N.S. involvement.

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REFERENCES

CASSELMAN, A. A., GREENHALGH, R., BROWNELL, H. H. & BANNARD, R. A. B. (1960). Clam poison: I. The paper chromatographic purification of clam poison dihydrochloride. *Can. J. Chem.* **38,** 1277–1290.

CHENG, K–K., LING, Y–L. & WANG, J. C–C. (1968). The failure of respiration in death by tetrodotoxin poisoning. *Q. Jl exp. Physiol.*, **53,** 119–128.

EVANS, M. H. (1964). Paralytic effects of "paralytic shellfish poison" on frog nerve and muscle. *Br. J. Pharmac. Chemother.*, **22,** 478–485.

EVANS, M. H. (1965). Cause of death in experimental paralytic shellfish poisoning (psp). *Br. J. exp. Path.*, **46,** 245–253.

EVANS, M. H. (1968). The cauda equina of the rat: a convenient 'desheathed' mammalian nerve preparation. *J. Physiol., Lond.*, **194,** 51–52*P*.

EVANS, M. H. (1969a). The effects of saxitoxin and tetrodotoxin on nerve conduction in the presence of lithium ions and of magnesium ions. *Br. J. Pharmac.*, **36,** 418–425.

EVANS, M. H. (1969b). Differences between the effects of saxitoxin (paralytic shellfish poison) and tetrodotoxin on the frog neuromuscular junction. *Br. J. Pharmac.*, **36,** 426–436.

FURUKAWA, T., SASAOKA, T. & HOSOYA, Y. (1959). Effects of tetrodotoxin on the neuromuscular junction. *Jap. J. Physiol.*, **9,** 143–152.

HALSTEAD, B. W. (1965). *Poisonous and Venomous Marine Animals of the World.* Vol. **1**: Invertebrates. pp. 994. Washington, D.C., U.S. Govt. Printing Office.

JACKIM, E. & GENTILE, J. (1968). Toxins of a blue-green alga: similarity to saxitoxin. *Science, N.Y.*, **162,** 915–916.

KAO, C. Y. (1966). Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmac. Rev.*, **18,** 997–1049.

KAO, C. Y. & FUHRMAN, F. A. (1967). Differentiation of the actions of tetrodotoxin and saxitoxin. *Toxicon*, **5,** 25–34.

MCFARREN, E. F. & BARTSCH, A. F. (1960). Application of the paralytic shellfish poison assay to poisonous fishes. *J. Ass. off. agric. Chem.*, **43,** 548–554.

MOLD, J. D., BOWDEN, J. P., STANGER, D. W., MAURER, J. E., LYNCH, J. M., WYLER, R. S., SCHANTZ, E. J. & RIEGEL, B. (1957). Paralytic shellfish poison. VII. Evidence for the purity of the poison isolated from toxic clams and mussels. *J. Am. chem. Soc.*, **79,** 5235–5238.

NEEDLER, A. B. (1949). Paralytic shellfish poisoning and *Gonyaulax tamarensis*. *J. Fish. Res. Bd Can.*, **7,** 490–504.

NISHIYAMA, A. & KAO, C. Y. (1964). Presynaptic actions of saxitoxin at neuromuscular junction. *Fedn Proc.*, **23,** 298.

PRAKASH, A. (1963). Source of paralytic shellfish toxin in the Bay of Fundy. *J. Fish. Res. Bd Canada.* **20,** 983–996.

RIEGEL, B., STANGER, D. W., WILKOLM, D. M., MOLD, J. D. & SOMMER, H. (1949). Paralytic shellfish poison. V. The primary source of the poison, the marine plankton organism, *Gonyaulax catenella*. *J. biol. Chem.*, **177,** 7–11.

SAKAI, F., SATO, A. & URAGUCHI, K. (1961). Über die atemlähmung durch tetrodotoxin. *Naunyn, Schmiedeberges Arch. exp. Path. Pharmak.*, **240,** 313–321.

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

SCHANTZ, E. J. (1967). Biochemical studies on purified *Gonyaulax catenella* poison. In *Animal Toxins*, ed. Russell, F. E. & Saunders, P. R., pp. 91–95, Oxford & New York: Pergamon Press Ltd.

SCHANTZ, E. J., MOLD, J. D., HOWARD, W. L., BOWDEN, J. P., STANGER, D. W., LYNCH, J. M., WINTERSTEINER, O. P., DUTCHER, J. D., WALTERS, D. R. & RIEGEL, B. (1961). Paralytic shellfish poison. VIII. Some chemical and physical properties of purified clam and mussel poisons. *Can. J. Chem.*, **39,** 2117–2123.

SCHANTZ, E. J., MOLD, J. D., STANGER, D. W., SHAVEL, J., RIEL, F. J., BOWDEN, J. P., LYNCH, J. M. WYLER, R. S., RIEGEL, B. & SOMMER, H. (1957). Paralytic shellfish poison. VI. A procedure for the isolation and purification of the poison from toxic clam and mussel tissues. *J. Am. chem. Soc.*, **79,** 5230–5235.

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.

SCHANTZ, E. J., LYNCH, J. M., VAYVADA, G., MATSUMOTO, K. & RAPOPORT, H. (1966). The purification and characterization of the poison produced by *Gonyaulax catenella* in axenic culture. *Biochemistry, N.Y.*, **5**, 1191–1195.

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

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

WEIL, C. S. (1952). Tables for convenient calculation of median-effective dose (LD50 or ED50) and instructions in their use. *Biometrics*. **8**, 249–263.

WOOD, P. C. (1968). Dinoflagellate crop in the North Sea. *Nature, Lond.*, **220**, 21.

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