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THE TOXIN FROM *GYMNODINIUM VENEFICUM* BALLANTINE

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(Text-figs. 1–8)

One of the authors (Ballantine, 1956) has recently described a new species of toxic dinoflagellate (*Gymnodinium veneficum*). Interest in the toxicity was stimulated by Bainbridge's observations (1953) on this species and by the well-known problem of mass mortality associated with 'red tide' outbreaks of poisonous flagellates. Much has been published on this latter subject, for which extensive bibliographies are available in the papers of Brongersma-Sanders (1948) and Hayes & Austen (1951).

Dinoflagellates in particular have long been associated with marine fish mortalities (see Davis, 1948, on *Gymnodinium brevis*, and the review by Nightingale, 1936) and with the problem of paralytic shellfish poisoning (Sommer, Whedon, Kofoid & Stohler, 1937, *Goniaulax catenella*; Koch, 1939, *Pyrodinium phoneus*; and Needler, 1949, *Goniaulax tamarensis*).

The published work on the mode of action of dinoflagellate toxins has been mainly concerned with the practical aspects linked with the cause, effects and prevention of paralytic shellfish poison, and has been directed towards mammalian and amphibian tissues (e.g. Kellaway, 1935; Fingerman, Forester & Stover, 1953), with attempts to ascertain the chemical nature of the poisons and with bio-assay. As there is no 'red tide' problem here, we are not concerned with the essential practical preventative approach and have attempted, over a wide field, to determine the actual site and mode of action of the toxin in marine animals, in addition to trying to extract it and find the effect on whole animals.

All our experiments have made use of a uni-algal culture of *Gymnodinium veneficum* grown in Erdschreiber culture solution (Plymouth formula).[1] These cultures are not bacteria-free, but repeated attempts to grow the bacteria from these cultures in a variety of marine media have always resulted in non-toxic cultures. In view of this we feel confident that the toxin is either a product of the *Gymnodinium* itself or possibly a product of the action of bacteria on a waste substance produced by the *Gymnodinium*. This latter possibility does not seem probable, as the substance is an exotoxin secreted

[1] Erdschreiber. Filtered sea water 1 l., pasteurized. Soil extract (*w/v*) 50 ml., $NaNO_3$ 0·3 g, $Na_2HPO_4 . 12H_2O$ 0·02 g.

into the water, and whereas about 90% of the total toxin in any culture is present in the supernatant fluid, on a volume to volume basis the cells contain about 200–500 times as much toxin as the water. In addition, when the bacterial population of a culture becomes high the toxicity decreases.

In this research we had assistance and advice from many people. We cannot express our thanks to all personally, but in particular we mention the folowing: Dr Mary Parke for providing large volumes of *Gymnodinium* culture; Dr G. Y. Kennedy and his assistants of Sheffield University who have carried out all experiments using mice; Dr N. R. Stephenson of the Department of National Health and Welfare of Canada for a supply of paralytic shellfish poison; Dr J. P. Quilliam of St Bartholomew's Hospital, London, for carrying out experiments with isolated ganglia; Dr D. B. Carlisle of this laboratory for assistance with experiments on isolated hearts and much helpful discussion; Dr E. D. S. Corner, International Paints Research Fellow at Plymouth, for experiments on respiration rates, for a great deal of help and advice on extraction techniques and for continued encouragement; and to the Director and staff of the Plymouth Laboratory, especially workshop staffs, for their unfailing interest in this work, and the ready production of apparatus when required.

EXTRACTION

In studies on the effect of the *Gymnodinium* toxin on whole marine animals, whole cultures of the flagellate or supernatant water after the removal of the cells were used. The specific action of the toxin, however, could be investigated only on isolated tissues from animals and the use of sea water as a bathing medium severely limits the range of material. Extraction of the toxin in a more concentrated form free from salt therefore became necessary.

Most of the work on paralytic shellfish poison has been based on extraction from the digestive glands of poisonous but living shellfish. The procedure generally adopted in this extraction has been to mince the digestive glands and extract the toxin with acidified alcohol (4 ml. conc. HCl/l. alcohol), centrifuge, evaporate to dryness and wash with chloroform (Müller, 1935). The method has since been extended by other workers and purer extracts have been produced (Sommer & Meyer, 1937; Sommer, Monnier, *et al.* 1948; Sommer, Riegel, *et al.* 1948; and Riegel, *et al.* 1949*a*).

In view of the concentration of toxins from *Goniaulax* spp. by shellfish we fed *Mytilus edulis* with cultures of *Gymnodinium veneficum* in an attempt to obtain a similar effect. Strong cultures were lethal within a day and more dilute cultures eventually killed the *Mytilus* without any significant concentration of the toxin by the animal.

It was therefore necessary for us to extract the toxin from cultures. The methods of Sommer *et al.* referred to above have been extended to extraction

from the cells of *Goniaulax catenella* (Sommer, Whedon, *et al.* 1937; Riegel *et al.* 1949*b*). We followed this method using centrifuged cells of *Gymnodinium veneficum* and dissolved the solid residue in the appropriate bathing media for experiments on isolated tissues. Extracts prepared by this means were also dissolved in sea water, and it was found that immersion of fish in this solution did not give the same symptoms as the original culture (see p. 176). The cells were therefore extracted with neutral alcohol and under these conditions the characteristics of the original culture were completely retained. In all our work we find that 'acid extraction' alters the toxin and we have employed 'neutral extraction' in all cases except when stated. Hashimoto & Migita (1952) have noted a similar effect.

Extraction from cells only is very wasteful as most of the poison remains in the supernatant fluid, and large volumes of culture were needed in order to obtain significant quantities of cells. The supernatant fluid contains about 90% of the toxin in most cultures, but the problem is to separate it from the salt. Adsorption on carbon columns was attempted. Owing, however, to the large amount of salt present it was necessary to use a very active charcoal to adsorb the toxin in reasonable amounts. Elution from such carbon was very difficult; hot ethyl alcohol containing 5% pyridine or hot dioxan were the only successful eluants. These were removed by evaporation under reduced pressure. Owing to the variability between batches of carbon and to the difficulty of removing final traces of solvents dialysis was tried. Cell-free culture fluid was dialysed in tubes and the salt had gone in 6 h, leaving behind the toxin which was then evaporated to dryness under reduced pressure. Very little toxin is lost through the membrane indicating that the toxin molecule is large, probably of mol.wt. > 1000. By this process an active extract was obtained, although the product was contaminated by impurities from the soil extract used in growing the cultures. Work on the purification of these extracts will be attempted, but it is obvious even at present that this is a very potent poison.

EXPERIMENTS WITH WHOLE ANIMALS

Throughout this series of experiments marine animals were taken from the aquarium sea water and placed either in whole cultures of *Gymnodinium veneficum* or in the supernatant culture fluid from which the cells had been removed. Experiments with other non-toxic flagellates have shown that this treatment produces no ill-effects.

Various animals have been used (see Table 1) and they appear to fall into three main groups, as follows:

(A) *Not affected.* Animals of only one group, the Polychaeta, have been found to be completely unaffected by the toxin. Specimens of *Nereis diversicolor* and *Arenicola marina* survive in cultures apparently indefinitely. With

TABLE 1

Animal	Mode of treatment	Reaction group (see p. 171)	Time to death
COELENTERATA			
Calliactis parasitica	Imm., Imm. + inj.	B	2–3 days
Anemonia sulcata	Imm., Imm. + inj.	B	3–4 days
Ephyrae of *Aurelia aurita*	Imm.	C	< 1 min
ANNELIDA: POLYCHAETA			
Nereis diversicolor	Imm.	A	—
	Imm. + inj.	A	—
Nereis diversicolor	Imm. + inc.	A	—
Arenicola marina	Imm.	A	—
ARTHROPODA: CRUSTACEA			
Calanus finmarchicus (see Marshall & Orr, 1955)	Imm.	B	1–2 days
Tigriopus fulvus	Imm.	B	1–3 days
Hemimysis lamornae (see Bainbridge, 1953)	Imm.	B	2–3 days
Macromysis flexuosus	Imm.	B	3–4 days
Palaemon serratus	Imm.	B	>4 days
Eupagurus bernhardus	Imm.	B	2–3 days
Carcinus maenas	Imm.	B	>4 days
Cancer pagurus	Imm.	B	3 days
MOLLUSCA			
Mytilus edulis	Imm.	B	<3 days
M. galloprovincialis	Imm.	B	<3 days
Pecten maximus	Imm.	C	1 h
Lasaea rubra	Imm.	B	>1 day
Buccinum undatum	Imm.	C	1 h
Aplysia punctata	Imm.	C	1 h
Eusepia officinalis (cuttle fish)	Imm.	C	3 h
ECHINODERMATA			
Asterias rubens	Imm.	B	3–5 days
Ophiothrix fragilis	Imm.	B	<3 days
Ophiocomina nigra	Imm.	B	<3 days
TUNICATA			
Ciona intestinalis	Imm.	B	3 days
CEPHALOCHORDATA			
Amphioxus lanceolatus	Imm.	C	45 min
PISCES			
Scyllium canicula (2 ft.) (dogfish)	Imm.	C	3 h
Gadus pollachius (3 in.) (pollack)	Imm.	C	18 min
Blennius gattorugine (blenny)	Imm.	C	<45 min
Trachinus vipera (lesser weaver)	Imm.	C	30 min
Gobius niger (goby)	Imm.	C	5–15 min
G. virescens (goby)	Imm.	C	5–10 min
Pleuronectes platessa (5 in.) (plaice)	Imm.	C	30 min
Ctenolabrus rupestris (wrasse)	Imm.	C	15–20 min
AMPHIBIA			
Rana temporaria (frog)	Inj.	C	Strong rapid paralysis kills in *c.* 30 h
MAMMALIA			
Mus musculus (mouse)	Inj.	C	2–4 min

Controls using sea water and non-toxic cultures were made in all experiments, and the control animals survived much longer and were still healthy when the test animals had all died.

Imm., immersion in culture; inj., injection of extract; inc., incision.

Nereis some animals were injected and others cut down the ventral surface. They were then placed in cultures but showed no ill effects.

(B) *Slowly affected.* In this category are various animals including members of the Coelenterata, Crustacea, Mollusca, Echinodermata and Tunicata. It is felt that with all these, except the Mollusca, which can close their shells protectively, the reason for the slow effect of the toxin is due to the fact that the large toxin molecule cannot penetrate the animal; for Dr Sheina M. Marshall (personal communication) reports that *Calanus* which ate a large number of *Gymnodinium* from strong actively growing cultures died within 18 h, whereas poor feeders survived some days. We have found that this applies also to the other crustaceans, except *Eupagurus* where the increased sensitivity may be associated with the soft body.

Ophiothrix and *Ophiocomina* both fragment when immersed in *Gymnodinium* cultures, and the arms are finally broken up into short segments leaving the body which then dies.

In the molluscs of group B, i.e. *Lasaea* (see Ballantine & Morton, 1956) and *Mytilus*, the mechanism for prevention of entry of the toxin is simply shell closure. Both are intertidal bivalves capable of withstanding long exposure to unfavourable conditions and they close up when immersed in this culture. Eventually, however, sufficient poison enters to kill them. In all animals of this group, isolated excitable tissues are quickly blocked by the toxin (see p. 179).

(C) *Rapidly killed.* This last group is by far the most interesting and with it most of the work on whole animals has been carried out. Into this section fall molluscs without an adequate shell closure mechanism, *Amphioxus*, all the fish, frogs and mice—the last two after injection. In the marine animals the primary site of entry of the toxin is probably through the gills and not orally, as the supernatant culture fluid from which all cells have been removed is as quickly lethal as the whole culture, even with continuous aeration, and evidence from feeding experiments with mice suggests that such rapid and lethal penetration of the toxin cannot take place through the gut.

Gobius virescens was used as the test animal for comparison of the toxicity of cultures and extracts because of its availability and sensitivity to the toxin. A strong culture kills the fish in 5–6 min, the death time being prolonged as the toxin strength is reduced, and a bio-assay method has been worked out on this basis (Fig. 1). The symptoms shown by all fish when immersed in cultures, supernatant fluid from cultures, cells resuspended in sea water, and all toxic extracts produced at pH 7–8·5, were identical. The immediate reaction of the fish was a violent attempt to swim away from the unpleasant medium. In the goby this violent swimming, either forwards or backwards, continued for about 2 min and then subsided. Intense vasodilation and a strong expansion of the chromatophores to produce a marked colour pattern occur at this stage. At the same time the balance control mechanism is upset, and the fish

floats on its side or even upside down. Breathing slows considerably from a normal rate of 75–90/min to about 45/min after 2 min. Soon after immersion breathing is interrupted by a spasmodic violent 'vomiting reaction', which continues until death. Breathing continues to slow down, turning to irregular gasping at long intervals (up to 20 sec) and there is a lack of sensory response.

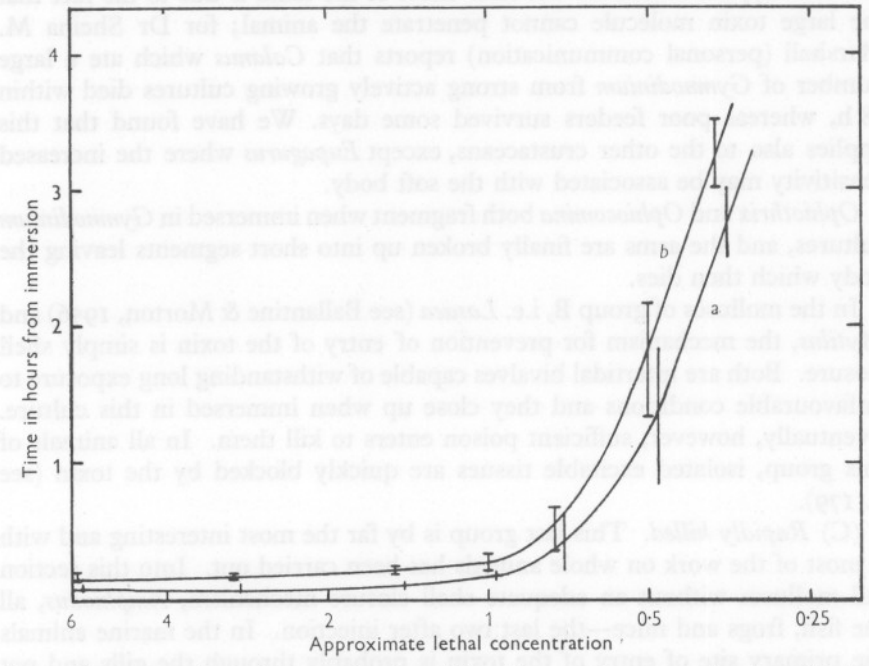

Fig. 1. Assay curve for toxin strength using gobies. Curve *a*, time to complete loss of balance in fish. Curve *b*, time to death. Extremes shown by vertical lines. Concentration in arbitrary units defined in text.

Reaction to touch or prodding disappears progressively from the head towards the tail, the base of the tail retaining sensitivity longest. Death occurs in quiescent paralysis, with the gills extended, apparently due to some sort of respiratory failure, but is preceded by a few irregular bursts of violent activity. If removed to clean sea water before the balance is upset the fish will recover; but there is no survival once the balance has gone.

The bio-assay technique we have developed using gobies is simple but only approximate. Fig. 1 shows the graphs obtained by plotting time to complete loss of balance (*a*) and time to death (*b*) against dose in arbitrary units. By using these two criteria it is possible to find an approximate lethal concentration per ml. of the sea water in which fish are immersed by using two or three different dilutions. This 'approximate lethal concentration' may be defined as the amount of toxin per ml. which causes permanent loss of balance

in 8–15 min and death in 10–20 min. A point which must be made about this assay is the fact that these times are constant with the gobies used (*G. virescens* and *G. niger*, where the loss of balance is interpreted as that stage when the fish can no longer right itself when inverted) regardless of the size of the goby. Fish from 17 mg to 7 g have been tested and body size has no effect on death time when a given dose of toxin is used. This is probably due to the fact that the toxin penetrates through the gills and the active gill surface area is roughly proportional to the size of the fish within a species. The average lethal concentration per ml. of a strongly toxic culture is about 6 units.

Because of its large size further experiments were carried out on the dogfish, and general symptoms were the same. Just before death (at the completely quiescent stage) the animal was removed and cut open. The heart was beating strongly, and there was no sign of haemolysis in blood samples taken. Electrical stimulation of the body muscles showed that these still responded to a direct stimulus. Although the muscles can themselves be poisoned by the toxin (p. 182), death is not due to failure of the muscles, and must result from a blockage in the nervous system.

Invertebrates of group C die with very few symptoms. Ephyrae of *Aurelia* pulsated about 5 times and then sank to the bottom fully contracted. Quick removal into clean sea water gave an eventual recovery in most cases. Molluscs died with their muscles relaxed, the bivalves with shells gaping.

During these experiments we have noted that, when agitated, the cultures produce an irritant vapour or mist (probably a suspension of droplets in air) which gives discomfort to throat and nose, of the type associated with an incipient cold. This phenomenon has also been observed as being associated with red tides in America (Woodcock, 1948; Ingle, 1954). One member of the staff of this laboratory, who is extremely susceptible to hay fever and allergic to many types of dust, is immediately and strongly affected by this mist. Since this type of reaction is associated with histamine release, several experiments were carried out on fish exposed to a variety of drugs. The histamine releaser '48–80' in sea water at strengths up to 100 μg/ml. has no effect at all on gobies. Histamine itself at 2·5 mg/ml. is not lethal but disturbs the animal and causes complete contraction of the chromatophores (greyish white) in contrast to the expansion in *Gymnodinium*. The addition of the antihistamine drug Phenergan at 2·5 μg/ml. to histamine-treated animals restored them to normal in a few minutes. On the other hand, Phenergan alone at 5 μg/ml. is lethal to gobies in 5 h. Phenergan gave no protection at all to gobies in *Gymnodinium* culture, and in fact hastened death. It therefore seems that there is no direct relation between the action of histamine and the toxin.

Evidence of protection against the toxin comes from two types of experiment: previous treatment in non-toxic flagellates, and addition of cholesterol. When gobies have been kept in cultures of non-toxic flagellates (e.g. *Isochrysis galbana* Parke) for 2 weeks, removed into clean sea water to clear the gills

of any possible clogging and then placed in *Gymnodinium* culture, time to death was prolonged from 15 min (control) to 2 hr. This protection was only slight. The influence of cholesterol was more dramatic, however. Cholesterol ground to a fine suspension in alcohol was added to sea water containing *Gymnodinium* culture. It was found that with a concentration of toxin of about 4 lethal units/ml (see Fig. 1), complete protection was provided if 20 mg. cholesterol was added per 100 ml sea water. Smaller doses prolonged the time to death. The actual amount of cholesterol which went into solution was uncertain.

Whereas extracts made at or near neutrality gave the same symptoms as the original culture, those made at pH 4, extracting from the centrifuged cells or from carbon columns with acidulated methyl alcohol (as described by Medcof *et al.*, 1947; and Müller, 1935), proved non-lethal to fish. The acid extract has a pronounced excitatory effect, does not slow breathing, and shows some resemblances to the action of acetylcholine. This difference in action between neutral and acid extracts is very marked. A similar observation has been made by Hashimoto & Migita (1952) on extracts from mollusc livers. Similar symptoms to these are shown by fish immersed in large doses of scallop liver extract (20 mg/100 ml.) which, if lethal, kills only after prolonged exposure (> 1 day).

All the experiments on mice reported below were done at the Cancer Research Department of Sheffield University by Dr G. Y. Kennedy and his assistants. Extracts were given to mice either orally or by intraperitoneal injection. Injection of neutral extracts in lethal doses caused death within 2 min, with immediate prostration and no other symptoms. There were no post-mortem aberrations. With sublethal doses the mice looked 'thoughtful' and slightly depressed, becoming moribund as the dose was increased. Eventual recovery was complete. Injection of the acid extract produced a slight excitation but was not lethal in the doses given.

We have been fortunate in obtaining a sample of lyophilized scallop liver extract containing the acid extract of paralytic shellfish poison from Dr N. R. Stephenson of the Department of National Health and Welfare of Canada (see also Stephenson *et al.*, 1955). This was also administered to mice during the series of tests. Injection of lethal doses produced prostration, and sub-lethal doses gave excitation. Oral administration also produces excitation of the animals, and is less toxic than injection. The *Gymnodinium* extracts were not toxic when administered orally, but again caused excitation. Thus it would appear that the neutral extract undergoes a change during digestion, probably due to the acid nature of the gastric juices, giving symptoms similar to those produced by acid extracts of *Gymnodinium* and by scallop liver extracts.

The evidence from fish experiments suggesting that death is due to respiratory failure is supported by injection of frogs. A quantity of neutral extract

sufficient to kill a mouse twice the weight was injected into the dorsal lymph sac of frogs. This caused immediate and complete paralysis, and the animals remained moribund for about 2 h. Some recovery then appeared to occur, but the animals died in 24–48 h. There was no sign of breathing over this period, but the oxygen supply through the skin must have been adequate to sustain life for this length of time. Prinzmetal, Sommer & Leake (1932) have also noted this greater resistance of frogs to paralytic shellfish poison.

Although death in vertebrates seems to be due to failure of the respiratory system to provide an adequate oxygen supply there is no doubt that the poison acts by blocking the nervous system.

EXPERIMENTS ON ISOLATED TISSUES

These were carried out to show the actual site of action of the toxin, and form a progressive series as information was accumulated. Unless it is otherwise stated, the tissues were treated with either the supernatant fluid from cultures if sea water was a suitable bathing medium, or neutral extracts of the toxin dissolved in an appropriate bathing fluid. 'Acid extracts' were used only in a few experiments, and a distinct difference in action was found in all of them, after which 'acid extraction' was abandoned in this project. The experiments will be grouped together under a series of general headings and for brevity only the salient points emerging will be discussed.

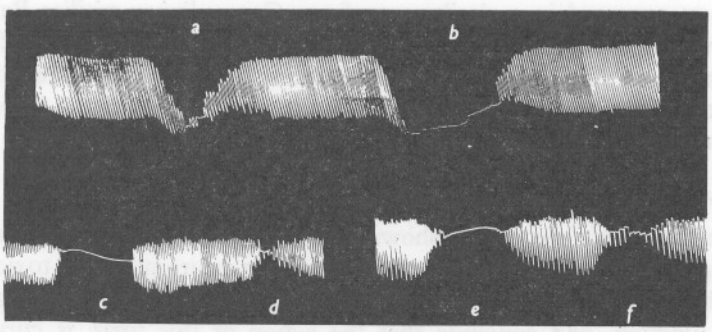

Fig. 2. Action of *Gymnodinium* toxin on perfused *Buccinum* heart. Contraction of heart (systole) downwards; perfusion fluid sea water; temp. 15° C. (*a*) Original culture diluted twofold; (*b*) neutral extract; (*c*) 'acid extract'; (*d*) 'acid extract' at half previous strength; (*e*) and (*f*) acetylcholine at concentrations of 5×10^{-7} and 2×10^{-7} respectively.

Isolated hearts.

Hearts from various animals were isolated and perfused. Recording was on a kymograph with a normal heart lever. Frog, *Loligo* and *Buccinum* hearts were all arrested in systole by the toxin, in contrast to the actions of acetylcholine and the 'acid extract', both of which cause arrest in diastole (Fig. 2).

Buccinum heart appears to be quite sensitive to the toxin and may be suitable for a comparative assay technique. The disadvantage of *Buccinum* heart when isolated and suspended is that the auricle is extremely friable, and the ligatures necessary for cannulation frequently sever it. A more reliable preparation results from cannulation of the heart *in situ*, via the efferent branchial vein. If a cannula with a long tip is used there is no need to ligate this vein. A small amount of citrate added to the perfusion fluid (sea water) prevents coagulation of the residual blood in the heart.

With *Maia* heart the 'acid extract' increases the heart rate whereas the neutral extract slows it.

Frog rectus abdominus muscle

Because of the similarity in action of the 'acid extract' and acetylcholine on isolated hearts we investigated the effect of our extracts on frog rectus abdominus muscle. The muscle was mounted in a chamber and connected to a simple lever which recorded on a kymograph. Bathing solutions were run into the chamber as necessary. The response to acetylcholine was first measured. Application of the 'acid extract' of the toxin caused a slow, steadily increasing contracture, but on the addition of acetylcholine the muscle contracted to the same final level as with acetylcholine alone. Relaxation of the contractured muscle is very slow. The neutral extract induces no contracture, but reduces the response of the muscle to acetylcholine, slowing the rate of contraction and almost stopping relaxation.

Extracts prepared in a similar way from *Gymnodinium vitiligo* Ballantine (1956), which is non-toxic to fish, were used on both hearts and muscles, and had no action at all. Thus it is assumed that any pharmacological reactions are due to the toxin and not to other cell products with which the extracts are undoubtedly contaminated.

Frog sartorius nerve-muscle preparation

Fingerman *et al.* (1953) have shown that paralytic shellfish poison extracted from digestive glands of poisonous shellfish is a neuromuscular toxin. They found that it eliminates the transmission of an impulse across the frog nerve-muscle junction, and conclude that the action of this toxin is curare-like. We have carried out similar experiments using neutral extracts of *G. veneficum*, and the preparation becomes inexcitable to stimulation through the nerve, without the nerve itself being affected. End-plate potentials can be recorded just after indirect excitability has disappeared, but these also decrease. The muscle does not respond to direct stimulation when the end-plate potential drops. In contrast to the conclusions of Fingerman *et al.* we find, as shown below (p. 182), that the membrane potential in the muscle has been destroyed and therefore the action of this toxin differs from that of curare. The 'acid extract' also renders the muscle inexcitable, but we have not followed this up.

Mechanical response of muscles.

The time course of the effect of the toxin on the mechanical response of frog and *Mytilus* muscles (Figs. 3, 4) shows that as the excitability decreases the rates of rise and fall of tension become slower.

The frog sartorius nerve-muscle preparation was mounted in a muscle bath and connected to an isometric lever. Tensions were recorded photo-electrically and displayed on a cathode-ray tube (Hill, 1949*a*). Stimulation could be applied to either nerve or muscle. When the anterior byssus retractor muscle of *Mytilus* was used the muscle was mounted on a multi-electrode assembly and again connected to the same recording apparatus (Abbott & Ritchie, 1951). Stimulation was by square wave pulses from an electronic stimulator.

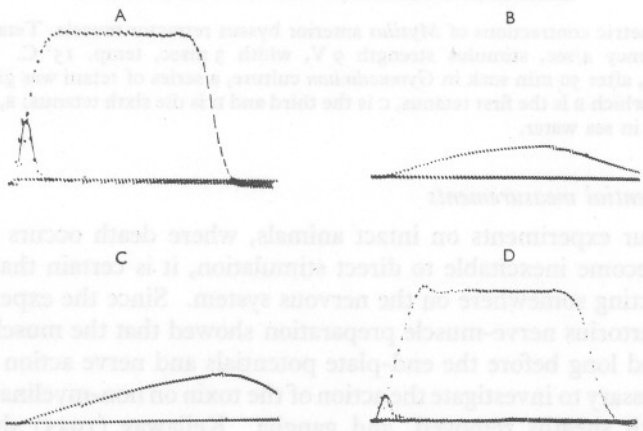

Fig. 3. Mechanical response of frog sartorius nerve-muscle preparation. Twitch and tetanic (25/sec) stimulation applied in each figure. Time base 1·5 sec, temp. 15° C. A, indirect maximal stimulation of untreated preparation; B, indirect stimulation after 10 min soak in neutral toxin extract; the twitch has disappeared; C, direct (muscle) stimulation immediately after B; D, indirect stimulation after 30 min soak out in normal Ringer.

Figs. 3, B, C show that even when the active isometric tension has been reduced to one third by the toxin (10 min immersion), the response by frog sartorius muscle to direct stimulation is identical with that to indirect stimulation, a fact not consistent with the action of a curare-like substance. It can also be seen that the twitch has by this time disappeared and that tetanic tension rises and falls very slowly. After 5 min soak-out in normal Ringer the tetanus tension had recovered to about 50% and was completely restored (Fig. 3D) in 30 min, together with the twitch.

Mytilus muscle (Fig. 4) similarly becomes inexcitable in the presence of the toxin. In a toxin-treated muscle successive tetani show facilitation (B, C, D) when stimulated by a voltage which gives a maximal response in the untreated

muscle (A). Increased voltage, however, gives a tetanus equivalent to that shown at the end of a series of facilitating tetani (D). Thus the voltage required to give a maximal response steadily increases as the toxin acts.

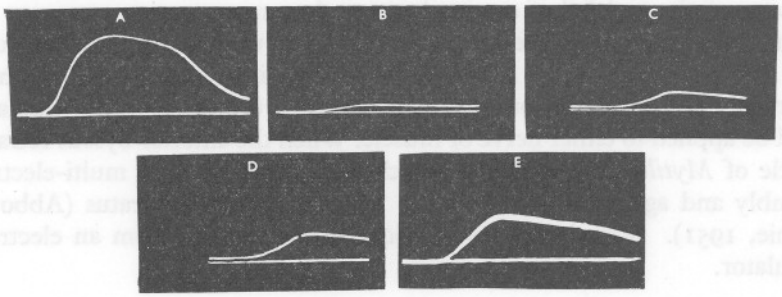

Fig. 4. Isometric contractions of *Mytilus* anterior byssus retractor muscle. Tetanus duration 5 sec, frequency 4/sec, stimulus strength 9 V, width 3 msec, temp. 15° C. A, untreated muscle; B–D, after 50 min soak in *Gymnodinium* culture, a series of tetani was given at 30 sec intervals, of which B is the first tetanus, C is the third and D is the sixth tetanus; E, tetanus after 3 h soak out in sea water.

Action potential measurements

From our experiments on intact animals, where death occurs before the muscles become inexcitable to direct stimulation, it is certain that the toxin must be acting somewhere on the nervous system. Since the experiments on the frog sartorius nerve-muscle preparation showed that the muscle response disappeared long before the end-plate potentials and nerve action potentials, it was necessary to investigate the action of the toxin on non-myelinated nerves, nerves with sheaths removed, and ganglia. Kellaway (1935) showed that sensory nerve endings in frog skin were quickly blocked by paralytic shellfish poison.

Lengths of nerve about 4 cm were dissected and mounted between forceps in an arrangement similar to that of Keynes & Lewis (1951), with the ends of the nerve higher than the central portion which was immersed in the bathing solution all the time. The whole assembly was on a Palmer stand so that the whole nerve could be immersed between records. A recorded action potential means that the whole immersed length of the nerve is conducting. Nerves were first soaked in their normal bathing solution and excitability checked. Toxin was then added and the effect with time noted.

Leg nerve of *Maia* (spider crab) was used as an example of a non-myelinated nerve with very little sheath (Fig. 5). The bathing solution was that described by Welsh (1936). Within 3 min of immersion in toxin the action potential had become spread out into a series of humps (B) and then steadily decreased in amplitude over about 45 min (C, D). Washing in normal Ringer restored the action potential.

When either ray wing motor nerve or frog sciatic nerve was tested the toxin was found to have only a small, slow effect on the intact nerve. When the nerve sheath is removed the nerves remain viable in normal Ringers (ray Ringer— Babkin, Bowie & Nicholls, 1933; frog Ringer— Hill, 1949*b*) for many hours, but conduction is quickly blocked in the presence of the toxin (less than 30 min). The inability of the toxin to block sheathed nerves is probably due to the fact that the molecule, which we believe to be large (p. 171), cannot penetrate the sheath.

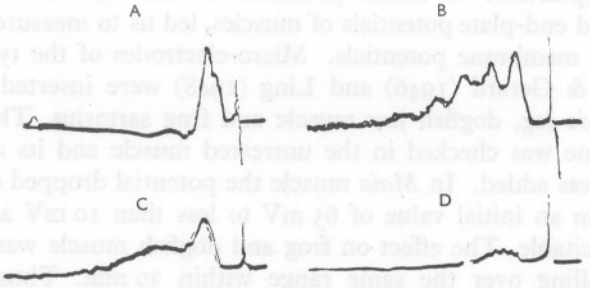

Fig. 5. Action potentials in *Maia* leg nerve. Time base sweep duration 150 msec. (right to left), temp. 15° C. A, untreated nerve; B, after 3 min in *Maia* Ringer with *Gymnodinium* toxin extract added; C, after 10 min in toxin; D, after 45 min in toxin.

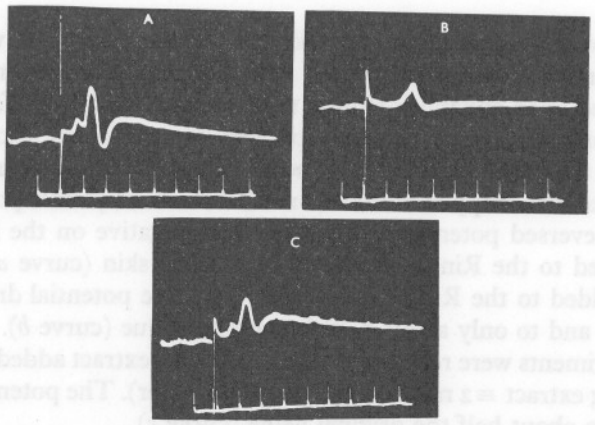

Fig. 6. Ganglionic potentials in isolated rat superior cervical ganglion. Time markers 50/sec, temp. 37° C. A, untreated ganglion; B, after 10 min in toxin; C, after 15 min soak out in normal Kreb's solution.

Ganglia

Preganglionic, ganglionic and postganglionic action potentials of isolated superior cervical ganglia of rats (at 37° C) were recorded for us by Dr J. P. Quilliam. Addition of toxin to the bathing solution slowly depressed the preganglionic potentials and more quickly depressed the postganglionic potentials,

both without appreciable change in their time course. The ganglionic potentials (Fig. 6), on the other hand, were depressed and eventually blocked, and their characteristics altered. In the untreated state there is an after-potential lasting nearly $\frac{1}{5}$ sec (A). As the toxin acts this after-potential is destroyed quicker than the spike (B). The potential returns to normal on soak-out of the toxin (C).

Membrane potentials

The disappearance of action potentials of nerves, and of both action potentials and end-plate potentials of muscles, led us to measure the effect of the toxin on membrane potentials. Micro-electrodes of the type described by Graham & Gerard (1946) and Ling (1948) were inserted into muscle fibres of *Maia* leg, dogfish jaw muscle and frog sartorius. The membrane potential value was checked in the untreated muscle and its change noted when toxin was added. In *Maia* muscle the potential dropped over a period of 1–2 h from an initial value of 65 mV to less than 10 mV and the fibres became inexcitable. The effect on frog and dogfish muscle was more rapid, potentials falling over the same range within 30 min. Thus, apparently, although the precise point at which the toxin acts to kill an animal is unknown, it functions by depolarising excitable membranes.

Frog skin

Further evidence that the toxin reduces cellular potentials was obtained with frog skin. Isolated pieces of frog skin taken from the abdominal surface were mounted in an apparatus similar to that described by Koefoed-Johnsen, Ussing & Zerahn (1952). The skin separated two chambers, each containing Ringer, and the potential difference across the skin was measured (Fig. 7). The skin potential dropped within 10 min from about 70 mV positive on the inside to a reversed potential of about 10 mV negative on the inside, when toxin is added to the Ringer on the inside of the skin (curve *a*). When the toxin was added to the Ringer outside the skin, the potential dropped much more slowly and to only about half the original value (curve *b*).

The experiments were repeated with scallop liver extract added to the inside Ringer (2 mg extract $\equiv$ 2 mouse units to 4 ml. Ringer). The potential again fell slowly and to about half the original value (curve *c*).

In all experiments the potential was restored to its original level within a few minutes of replacing the toxin with normal Ringer.

Ussing & Zerahn (1951) have explained the standing potential across frog skin in terms of an equivalent electrical circuit in which the sodium pump provides a driving potential in series with a resistance to the movement of sodium ions, with a passive resistance to other ions acting in parallel to this circuit. Disappearance of the potential could occur from a blockage of the sodium pump or from a greatly decreased passive skin resistance. A measure

of the skin resistance was obtained by forcing extra current through the skin from an outside source. The potential recorded across the skin varies with the current passed in a nearly linear manner and the slope of the potential–current relationship represents the effective total conductance of the skin. It was found that the skin conductance, and therefore the resistance, did not alter measurably when the skin potential was abolished by the toxin. It therefore appears that the toxin is acting on the sodium exchange mechanism. The brief description given recently by Koefoed-Johnsen & Ussing (1956) of a modified theory of the origin of frog skin potential is discussed later (p. 186).

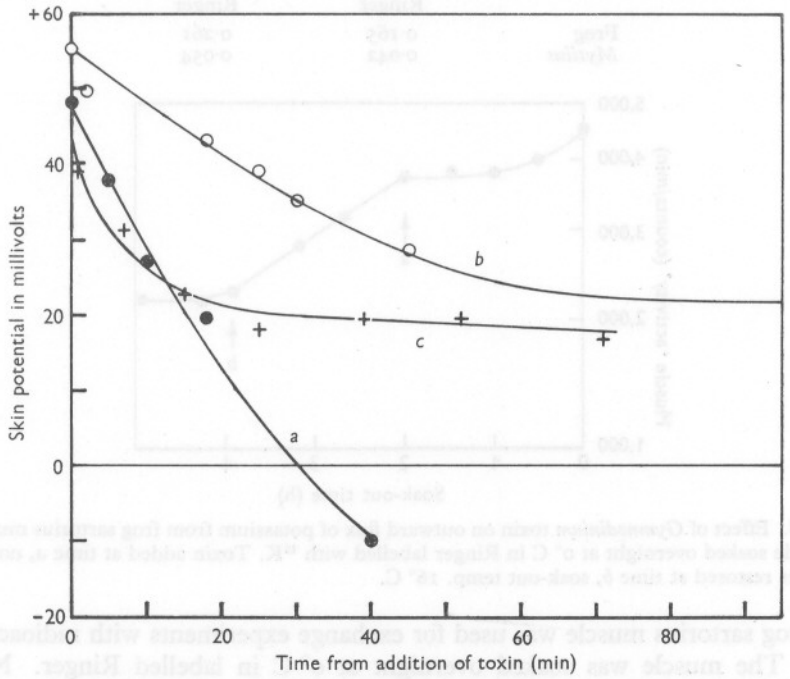

Fig. 7. Time course of decay of potential across isolated frog skin in the presence of toxins. Ringer on both sides of skin, temp. 15° C. Curve (*a*), neutral *Gymnodinium* toxin extract added to inside surface of skin; curve (*b*), neutral *Gymnodinium* toxin added to outside surface of skin; curve (*c*), scallop liver extract added to inside surface of skin.

Other experiments

Two other lines of approach have been made and preliminary results are quoted here, though further investigation is proceding.

The influence of the toxin on the rate of oxygen consumption by frog and *Mytilus* muscles has been measured, and we find that the rate is increased by 58 and 28% respectively (Table 2).

It is obvious that a great deal of information can be obtained from studying, with radioactive potassium and sodium, the influence of the *Gymnodinium*

toxin on the ionic exchanges across cell membranes. In resting nerve and muscle the membrane potential is closely related to the ratio of potassium concentrations on the two sides of the membrane. The speed with which the toxin acts, and also the rate of recovery, is surprisingly fast if the entire potential decrease is due to leakage of potassium out of the cell.

TABLE 2. RESTING RESPIRATION RATES OF FROG AND *MYTILUS* MUSCLE, AT 19·7° C

(μl O_2/mg wet wt/h)

	Normal Ringer	Toxin-treated Ringer
Frog	0·165	0·261
Mytilus	0·042	0·054

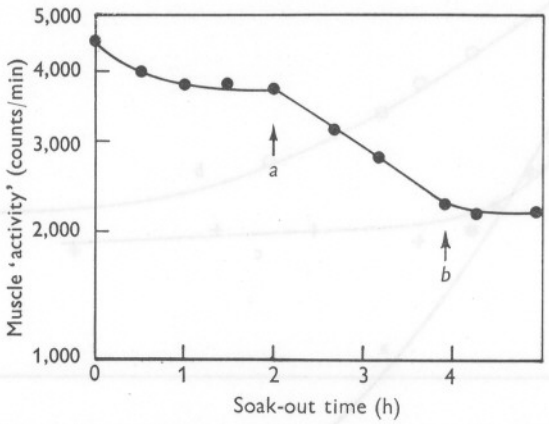

Fig. 8. Effect of *Gymnodinium* toxin on outward flux of potassium from frog sartorius muscle. Muscle soaked overnight at 0° C in Ringer labelled with ^{42}K. Toxin added at time *a*, normal Ringer restored at time *b*, soak-out temp. 16° C.

Frog sartorius muscle was used for exchange experiments with radioactive ^{42}K. The muscle was soaked overnight at 0° C in labelled Ringer. Next morning it was transferred to normal Ringer at room temperature to wash out the radioactivity. The activity of the muscle was counted at intervals as soaking out continued. When the extracellular potassium had diffused away, the activity (after correcting for decay of the isotope) decreased exponentially with time over several hours. The slope of the curve gives a measure of the potassium efflux, and the half-time of exchange (Fig. 8) is about 30 h. Toxin was then added to the soak solution, at the time indicated on the curve. The muscle was inexcitable within 20 min. The efflux rate of potassium increased greatly but the time for half exchange was about 3 h, so that the membrane potential disappeared long before the internal potassium level had fallen to the requisite value to account for the depolarization.

The experiments described in this section are summarized in Table 3.

TABLE 3. SUMMARY OF THE ACTION OF *GYMNODINIUM* TOXIN ON VARIOUS TISSUES

Preparation	Effect of 'Neutral' toxin extract	Page reference
Buccinum heart	Arrest in systole	177
Frog rectus abdominus	No contracture; reduction of sensitivity to acetylcholine: slowing of relaxation	178
Frog sartorius nerve-muscle	(1) indirect stimulation: muscle inexcitable, nerve slightly affected, end-plate potentials decrease and disappear. Muscle twitch quickly goes, tetanic tension decreases and becomes slower in rise and fall	178
	(2) direct stimulation: mechanical response decreases and is always identical with response to indirect stimulation	179
	Muscle respiration rate increased	183
	Efflux rate of potassium greatly increased	184
Mytilus anterior byssus retractor	Becomes inexcitable. Progressive threshold increase, mechanical response is slowed	179
Nerve, crab	Action potential spreads out into several humps, and disappears	180
Nerve, de-sheathed frog sciatic	Blocked	181
Superior cervical ganglion of rat	Ganglion potential depressed and then blocked. After-potential disappears first	181
Frog skin	Toxin on inside, potential decreases and reverses in sign. No measurable change in skin resistance. Toxin on outside, very slow partial drop in potential	182

DISCUSSION

Sommer & Meyer (1937) have suggested that the toxin produced by dinoflagellates and causing paralytic shellfish poisoning may be different from that liberated by other dinoflagellates associated with mass mortality of fish. Our observations on the toxin from *Gymnodinium veneficum* tend to confirm this view, as we are unable to render shellfish poisonous with it, and it differs from the poison in lyophilized scallop liver extract, both in action on fish (p. 176) and mice (p. 176) and on isolated frog skin (p. 182). Whereas Fingerman *et al.* have indicated that the paralytic shellfish poison operates in a curare-like manner, we have shown that the *Gymnodinium* toxin depolarizes the excitable membrane.

Our evidence from the frog skin, considered in the light of Ussing's original hypothesis of the origin of the skin potential, suggests that the toxin blocks the sodium pump—particularly as the skin resistance is not measurably altered. In the case of nerve and muscle, however, blockage of the sodium pump could only result in depolarization of the membrane after sufficient potassium had leaked out, in exchange for the sodium entering the fibre—a process which would take very much longer than is needed for depolarization with the toxin. In a similar way the recovery is much too rapid to be explained by a reversal of this process. Moreover, analysis of muscles which

have been rendered completely inexcitable by the toxin shows that they still contain considerable amounts of potassium. The tracer experiments mentioned above (p. 184) also show that although the potassium efflux increases in the presence of the toxin, the exchange is not rapid enough to explain the depolarization (in terms of a concentration cell).

Koefoed-Johnsen & Ussing (1956) have recently modified their hypothesis and now suggest that two separate membranes are concerned with the potential in frog skin: on the outer surface there is a membrane selectively permeable to sodium, and on the inner surface a membrane which is selectively permeable to potassium. A sodium-potassium exchange pump, located in the inner membrane, maintains a low sodium and high potassium concentration in the skin cells. The potential measured across the skin is the sum of the sodium diffusion potential across the outer membrane and the potassium diffusion potential across the inner membrane. Such a potential would decrease if the inner membrane became permeable to sodium ions; and if the permeability became greater than that of the outside membrane the potential could certainly temporarily reverse. We find that the toxin produces a reversal of potential. Certainly the selectivity of the toxin for the inside of the skin indicates that its site of action is there.

An increase in sodium permeability would also reduce the membrane potential in nerve and muscle cells. Such a permeability increase occurs during the rising phase of an action potential, when the potential drops rapidly and reverses in sign.

From these arguments we conclude that the *Gymnodinium* toxin is acting on the nervous system of animals by depolarizing excitable membranes. We feel that this probably occurs by interference with the sodium exchange mechanism. The speed of action of the toxin makes it improbable that it is only stopping sodium extrusion, and the more probable explanation is that it allows rapid entry of sodium into the cells.*

SUMMARY

Methods of extraction of the toxin from cultures of *Gymnodinium veneficum* are described. This is now done by dialysis and evaporation under reduced pressure. The toxin molecule must be large, as it cannot penetrate a dialysis membrane; it is soluble in water and the lower alcohols, but insoluble in ether and chloroform. It is unstable in acids, turning into another toxic product, and is decomposed by hot alkali, though in neutral solution is more or less thermostable. The toxin as it occurs in sea water is not the same as paralytic shellfish poison, but there are some resemblances between this and the 'acid extract', though much more work is needed to check this point.

The action of the toxin on a variety of animals is described, and an attempt

* See note at end of paper.

is made to devise an approximate assay technique using gobies as the test animals.

The action has also been observed on a range of isolated preparations, and although the final conclusion as to the mode of action is not quite clear, we feel confident that the site of action in whole animals is in the nervous system, probably acting on ganglion transmission. With regard to the mode of action it depolarizes nerve and muscle membranes. It also abolishes the potential across frog skin without measurably altering the skin resistance.

This depolarization probably occurs by interference with the sodium exchange mechanism, allowing rapid entry of sodium into the cells.

Note added in proof

Preliminary experiments have been carried out on the effect of this toxin on sodium permeability of frog sartorius muscle using the radioisotope ^{22}Na. Pairs of sartorius muscles were isolated. From each pair, one muscle was soaked in normal frog Ringer labelled with ^{22}Na and the other soaked in similar labelled Ringer to which toxin was added in sufficient quantity to render the muscle inexcitable. The exchange of sodium across the muscles was followed in terms of the radioactivity of the muscles: the muscles were removed at intervals, surface liquid blotted off and activity measured. Activity (and total sodium) of the toxin treated muscles increased more rapidly and to a higher final level than that of the untreated muscles. The tissues were later soaked in non-radioactive Ringer solutions and the decrease in activity followed. The activity of the normal muscle decreased rapidly at first corresponding to exchange in the extracellular sodium, and then more slowly as intracellular sodium exchanged. In the case of the toxin-treated muscle the exchange was very rapid and large, and consisted only of the one fast phase. It appeared that the membrane had become so permeable to sodium that the exchange from inside the cells had become similar in time course to that of the extracellular sodium.

The conclusions of this paper are of necessity preliminary and need verifying with a purified sample of the toxin. This, however, must await further development of the technique of producing large quantities of material and subsequent purification.

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