PHARMACOLOGY OF MARINE TOXINS AND CHOLINERGIC MECHANISMS

Thesis for the Degree of Master of Science

submitted by

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February 1971

CONTENTS

SUMMARY

A survey of the literature relating to venomous and poisonous species of Cnidaria and Mollusca is made. This includes a discussion of methods of envenomation, types of injuries caused by members of both phyla and problems of toxin isolation. The biological properties of several toxins are also discussed.

Some aspects of cholinergic mechanisms are surveyed. These include the structure of the mammalian motor end-plate and the events involved in neuromuscular transmission. Effects of chronic denervation on mammalian skeletal muscle are also described.

The experimental section of the thesis includes a pharmacological investigation of toxins isolated from the cnidarian Chironex fleckeri and the octopus Octopus maculosus as well as a study of facilitatory drug action at the neuromuscular junction.

Chironex fleckeri toxin can be separated into two lethal fractions with molecular weights of 70,000 and 150,000. The lower molecular weight fraction also possesses haemolytic and dermatonecrotic activity.

Both fractions produce an increase in arterial pressure in anaesthetized rats and rabbits by direct vasoconstriction. This is followed by hypotension, bradycardia and cardiac irregularity. Animals frequently show arterial pressure oscillations with alternate periods of apnoea and hyperpnoea; death results from respiratory arrest of central origin associated with marked signs of cardiovascular failure.

Experiments in which the vasomotor reflex pathway is interrupted by acute nerve section or drug blockade suggest that both toxins act partly by baroreceptor stimulation and/or depression of the brain stem vasomotor centre.

Both toxin fractions produce coronary vasoconstriction and a decrease in heart rate and amplitude of contraction in the isolated perfused guinea pig heart.

Octopus maculosus toxin (maculotoxin), which has similar pharmacological properties to tetrodotoxin and saxitoxin, causes hypotension, bradycardia and respiratory paralysis in anaesthetized rats and rabbits. The hypotension can be temporarily reversed with l-adrenaline or l-noradrenaline.

Respiratory failure is due to blockade of motor nerve axons. The toxin blocks transmission in rat and toad sciatic nerve and at low concentrations appears to have neuromuscular blocking activity. At higher concentrations the muscle membrane also becomes inexcitable.

Animals can be resuscitated after a marginally lethal dose by artificial ventilation alone, provided this is commenced before hypoxia becomes severe.

Facilitatory drug action has been studied in the phrenic nerve-diaphragm and the chronically denervated diaphragm of the rat. The latter preparation has been used in an attempt to study postsynaptic effects of the compounds.

The drugs used, tetrahydro-4-aminoacridine and a series of hydroxyanilinium compounds including

edrophonium, all produce twitch potentiation and spontaneous activity in the innervated preparation. These effects are depressed by temperature reduction, low Ca^{++} solutions or high Mg^{++} solutions.

The ACh contraction of the denervated diaphragm was potentiated by low concentrations of all drugs except 3-hydroxyphenyltriethylammonium; the ACh depolarization was similarly affected. Potentiation was suppressed by raised levels of Ca^{++} or Mg^{++}.

Interactions between the facilitatory drugs and succinylcholine are described.

Although postsynaptic action is likely to be a contributing factor in the mechanism of action of these facilitatory drugs, the results suggest that facilitation is largely a presynaptic event.

D E C L A R A T I O N

The studies described in the experimental sections of the thesis have been reported in several papers published by Dr. S.E. Freeman and myself.* The investigations have been conducted jointly with Dr. Freeman and largely under her direction. I have been solely responsible for the instrumentation of the many techniques employed and have participated equally in the experimental work involved. Preparation of the manuscript of the Turner and Freeman paper (see below) was my responsibility; I also assisted in preparation of the other manuscripts.

I declare that the thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

R.J. TURNER

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I would like to express my sincere thanks to Dr. S.E. Freeman for her advice and untiring encouragement throughout the writing of this thesis and Dr. T.E.B. Keen for his valuable criticisms of the sections on marine toxins.

I am also grateful to Mrs. M. Lovinger for her immaculate typing of the final draft of the manuscript.

LITERATURE SURVEY

PART A CNIDARIAN TOXINS

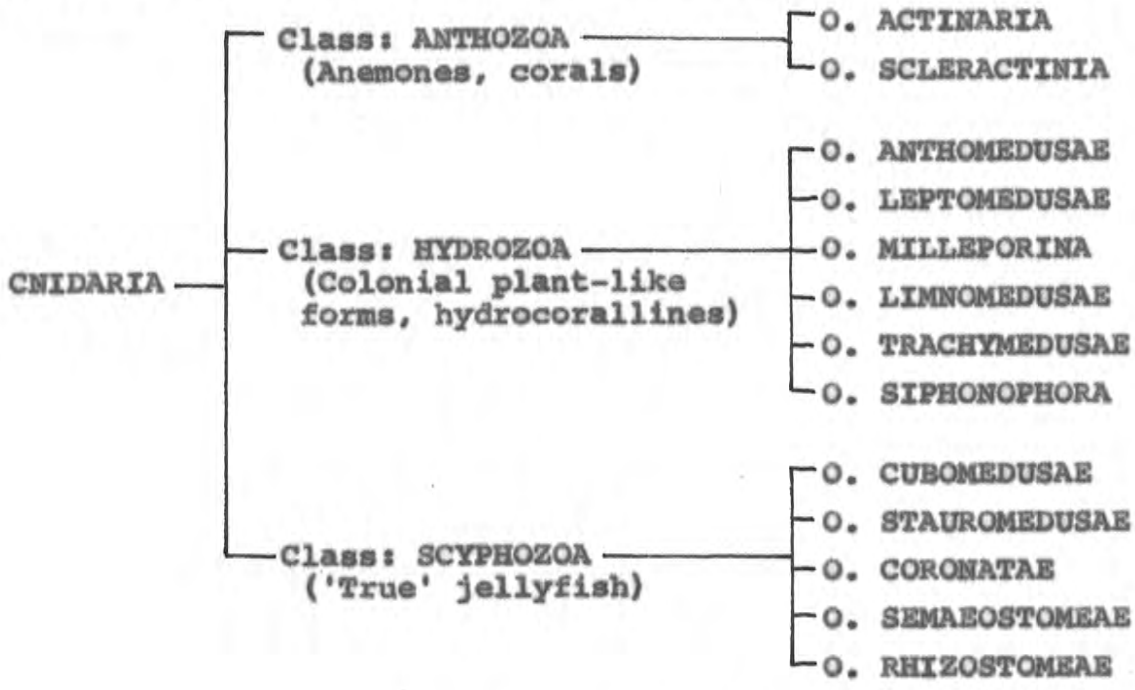
INTRODUCTION

There are more than 9000 species in the phylum Cnidaria. Corals, anemones and jellyfish, existing as solitary or colonial forms are all members of this invertebrate group, which is the lowest on the evolutionary scale to have definite tissue types. All of the Cnidaria are aquatic and most are marine, with species existing at all latitudes and from the littoral to the abyssal zone.

The basic cnidarian forms are the polyp and the medusa. The polyp has a tubular body, one end of which is closed and attached to the substrate, the other with a central mouth which is usually surrounded by a ring of tentacles. The medusa is a free-swimming, radially symmetrical form with a gelatinous umbrella, often with marginal tentacles. Table 1 shows the classification within the phylum. Further details of the anatomy and ecology of the various classes can be found in works by Thiel (1966), Barnes (1966), Southcott (1956), Lentz (1966), Hadži (1963), and Cleland and Southcott (1965).

Amongst their great diversity of form and adaptation, the one unique and distinctive feature which is characteristic of all cnidarians is the possession of nematocysts, minute structures which function both as a defence mechanism and a means of capturing prey.

Table 1. Taxonomic classification within the phylum Cnidaria.



N E M A T O C Y S T S

The nematocyst ranges in size from five microns diameter up to 1.12 x 0.12 mm for Halistemma rubrum. The classification of nematocyst types is complex with twenty basic types being categorized by Weill (1934), but for the purposes of this study these can be differentiated into two functional types:

(a) the non-injectors, or astomocnides, which may be either adhesive (Rhopalonemes) or springlike (Desmonemes). The function of both of these subtypes is to entangle and hold the prey.

(b) the injectors, or stomocnides, which inject a toxic material into the prey. The fact that this toxin is capable of causing injury and even death in humans explains why the Cnidaria have become the subject of considerable chemical, pharmacological and clinical research.

The nematocysts occur in greatest concentration in

small outpouchings or batteries in the ectodermal tissues of the tentacle. Each nematocyst develops from a vacuole within the Golgi apparatus of a cnidoblast cell or nematocyte (Chapman and Tilney, 1959; Hess, 1961; Slautterback, 1961; Picken and Skaer, 1966; Lentz, 1966). The mature nematocyst finally occupies most of the cell, pushing the nucleus to one side of the almost structureless cytoplasm. Tubules differentiate from the capsular matrix and spines and stylets develop according to the nematocyst type.

The threadlike tube which forms the injector mechanism is coiled regularly inside the nematocyst capsule. The stimulus required for discharge of the thread is not completely understood. In some Cnidaria discharge may occur after mechanical stimulation of a trigger hair or cnidocil which protrudes from the capsule (Kingston and Southcott, 1960; Lane, 1960), however in others the presence of a cnidocil has not been demonstrated (Hand, 1961) and in these other mechanisms must be operative. These could include chemical (McNeill and Pope, 1943b), surface active (Kingston and Southcott, 1960) or neural (Lentz and Wood, 1964) controls, but conclusive evidence favouring any one of these mechanisms is lacking.

The thread which, in the injectors, usually possesses spiral rows of barbs on its surface, discharges from the capsule by eversion (Picken and Skaer, 1966; Robson, 1953). This means that the tip of the shaft is formed by a constantly renewed front of opposed barbs which, in some species, provides an injector mechanism with sufficient force to penetrate fish scales and human skin (Lane, 1968a; Russell, 1967; Robson, 1953). The discharged thread is usually much longer than would be expected from observation of the intact nematocyst. Light microscopy studies (Picken, 1953) on Corynactis viridis suggested that the necessary volume

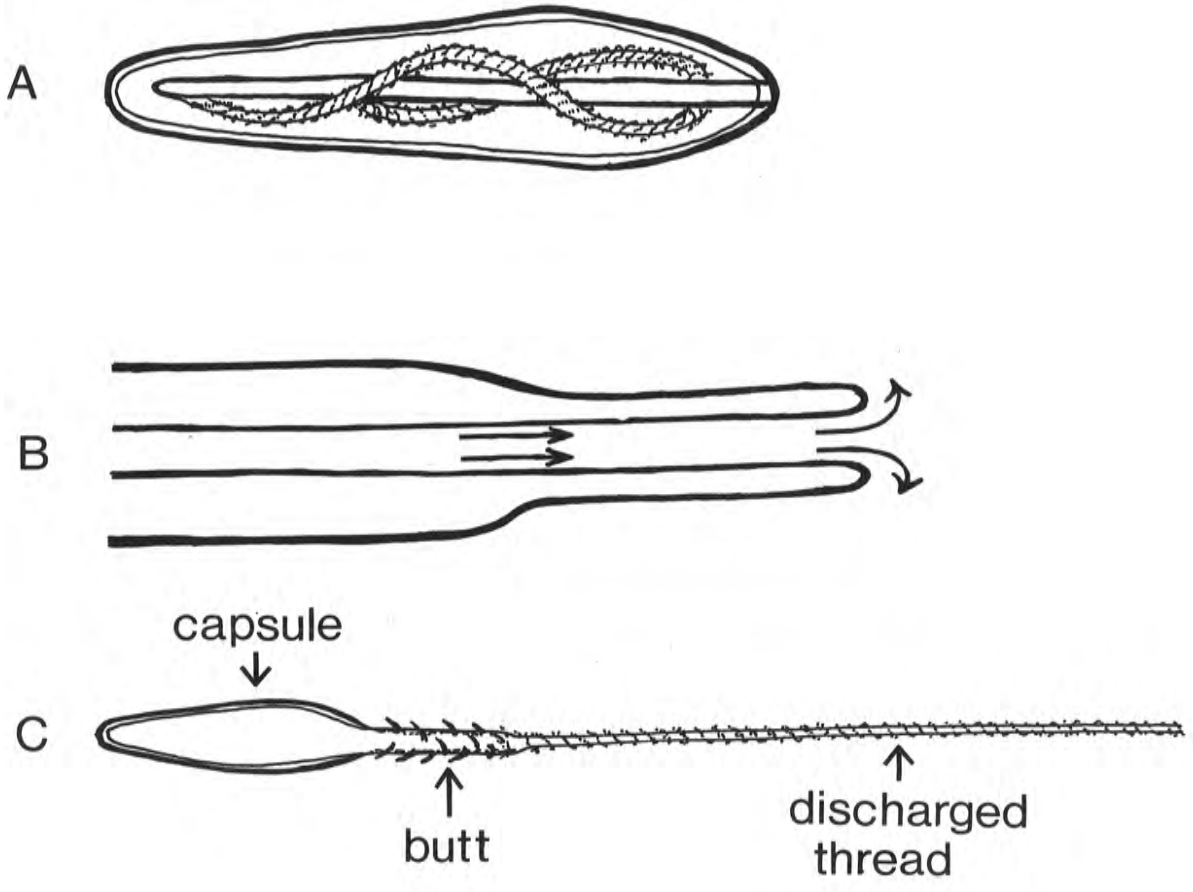


Figure 1: Illustration of nematocyst discharge.
A. Undischarged capsule showing thread coiled inside.
B. Eversion of thread. C. Discharged nematocyst showing empty capsule, butt and discharged thread with spiral rows of barbs.

increase occurred by anisometric swelling of the thread which became hydrated during eversion. However, some interesting electron microscopy by Picken and Skaer (1966) has shown that the undischarged thread is actually pleated into a screw shape which unfolds on discharge to become a smooth, tapered tube with the same surface area. Figure 1 illustrates the discharge of a typical stomocnide. It is not clear whether the toxin is injected through the discharged tube or carried on the discharging thread as it everts thus being deposited along the injection path. The latter mechanism would seem to be the more likely as well as the more effective way of introducing the toxin.

No information on the site of venom synthesis in the Cnidaria is available. The venom may be produced within the cnidoblast and incorporated into the nematocyst at an early stage of development or, as Lane (1961) speculates for Physalia, it could be produced at some more distant site and later incorporated into the mature nematocyst. The former suggestion is more acceptable especially when the relative impermeability of the nematocyst capsule and the diffusion problems associated with transfer of the high molecular weight toxins from Chironex fleckeri, Condylactis gigantea and Physalia physalis are considered.

INJURIES CAUSED BY MEMBERS OF THE PHYLUM CNIDARIA

More than seventy species of Cnidaria have been reported to be harmful to man. Although the most dangerous types are those which are capable of injecting venom by nematocyst discharge into the skin, there are a few reports of severe systemic effects and death following ingestion of a poisonous species. Painful injuries may also result from contact with several types of sharp corals.

Biologically active compounds have been demonstrated in

species which are not responsible for stingings in humans. It should, however, be remembered that the nematocyst is primarily a mechanism for capturing small crustacea or fish for food and in most species the nematocysts are either incapable of penetrating human skin or contain a toxin harmless to man in the amounts injected. The following account is a brief description of the types of injuries sustained and the species reportedly involved.

Class: Anthozoa

O. Actinaria. Anemones do not represent an important group when considering injuries to humans. Effects are confined in most cases to cutaneous blisters or rashes, slight to moderate pain and itching. One of the more serious injuries, the so-called 'sponge divers' disease', occurs throughout the Mediterranean and is caused by the anemone Sagartia elegans which attaches itself to the sponge. These stingings result in the general symptoms described and are usually followed by necrosis of the skin and ulceration which may leave permanent scarring.

Several other species listed by Halstead (1959) and Cleland and Southcott (1965) are capable of inflicting mild but sometimes painful stings. The anemone Rhodactis howesii has not been implicated in stingings but it may be toxic if eaten raw (the species is apparently cooked and eaten by some Pacific natives). Extracts of this species are lethal to toads (Martin, 1960) and mice (Farber and Lerke, 1963) but the lethal properties are destroyed by heating above 65°C. Both authors suggest that the material is a protein but its location within the anemone is unknown. The anemone Condylactis gigantea has been shown to contain a neurotoxin which prolongs nerve action potentials so that a type of depolarizing block is produced (Shapiro, 1968a,b; Shapiro and Lilleheil, 1969; Narahashi, Moore and Shapiro, 1969). Crayfish are very susceptible to this toxin which is not reported to be harmful to man.

O. Scleractinia. Injuries which result from abrasion by the calcareous outer skeleton of corals are often greater than can be explained solely by mechanical trauma. The wound is very susceptible to infection and healing is slow. Both the slime which covers living coral and nematocyst discharge from the polyp tentacles have been suggested as the agent responsible but no conclusive evidence is available.

Class: Hydrozoa

Several species are capable of causing skin lesions, wealing, rashes and stinging pains in man. Mild systemic effects including abdominal pain, chills, fever and diarrhoea have also been reported. The types responsible are described by Cleland and Southcott (1965) and Southcott (1963) and include members of all orders except the Trachymedusae.

Clinically, the most important group are siphonophores of the genus Physalia, which have been implicated in human stingings but as yet have not been positively identified as lethal. Physalia, the Portuguese 'man-of-war', is a colonial form in which the float or pneumatophore is one highly specialized unit, with other individuals modified to form fishing tentacles (dactylozooids), feeding forms (gastrozooids) and structures bearing male and female reproductive bodies. Two species are usually described, the Indo-Pacific form, Physalia utriculus, which has one main tentacle, and the Atlantic form, Physalia physalis, which has many. Current opinion, however, is that P. utriculus and P. physalis are the same species and both should be referred to as Physalia physalis (W.J. Rees, in Barnes, 1966). Descriptions of human stingings can be found in articles by Russell (1966), Cleland and Southcott (1965) and Barnes (1960). The stings are moderately to severely painful and are accompanied by wealing along the area of contact with the tentacle. Contact with a small specimen may result in no other effects but extensive stingings by larger Physalia may also produce weakness, nausea,

muscle spasm and difficult or painful respirations.

A considerable volume of research on Physalia toxin has been published by Lane and his coworkers who report that toxin liberated from isolated, washed nematocysts is a protein complex which is lethal to all multicellular animals tested. The toxin decreases the active transport of ions, especially potassium, against electrochemical gradients thus reducing or modifying the capacity of tissues and organs to generate bioelectric potentials (Lane, 1967). The properties of Physalia toxin will be described more fully later.

Class: Scyphozoa

Members of all orders except the Stauromedusae have been involved in human stingings. Identification of the agent responsible has often proved difficult as many jellyfish are practically transparent and others are very small. Difficulties also arise because common names often refer to different species in different regions. An exhaustive survey of all recorded stingings attributed to the Scyphozoa in the Pacific region is found in Cleland and Southcott (1965) and it is only relevant here to mention the most important species.

O. Semaeostomeae. This group is usually found in temperate regions and several species can inflict severe stingings. These include Dactylometra quinquecirrha and Cyanea capillata (Halstead, 1959; Cleland and Southcott, 1965; Barnes, 1960). Stings by both species are moderately to severely painful; Dactylometra may also produce symptoms of mental depression. Systemic effects due to Cyanea include nausea, muscular pain, mucoid hypersecretion throughout the respiratory tract, respiratory oppression and, in severe cases, loss of consciousness (Southcott, 1963).

O. Cubomedusae. Named because of their cuboid umbrella these jellyfish are found in tropical and subtropical waters. The order includes Chironex fleckeri (Southcott, 1956) and Chiropsalmus quadrigatus, the only cnidarians to which fatal

human stingings can be positively attributed. The toxins isolated from these species are among the most potent animal toxins known. Specimens of Chironex, which are almost invisible in the water, may measure up to 20 cm across and weigh 2.5 to 3 kg. Four pedalia at the corners of the umbrella give rise to several tentacles, the number and length depending on the size of the jellyfish. The tentacles may reach a length of 8 to 10 metres in the normal uncontracted state, representing more than 100 metres of tentacle in a mature specimen. Barnes (1966) suggests that, on the basis of tentacle size assuming an even distribution of nematocysts, Chironex fleckeri should possess 100 to 200 times as much venom as Chiropsalmus quadrigatus and thus is likely to be more dangerous. The two species together have been implicated in over sixty deaths and many more non-lethal stingings in Australian tropical waters.

Non-lethal stingings are painful with multiple lines of wealing. Epithelium on the crest of these weals carries a tranverse bar pattern corresponding to the closely spaced tentacular rings of nematocysts. The nematocyst threads penetrate through to the dermis (Kingston and Southcott,1960). Stings from larger specimens may cause massive oedema, deep necrosis and slow healing with permanent scars. Lethal stings produce intense pain and then sudden collapse preceded by a brief spasm of violent twitching. Post mortem examinations reveal pulmonary oedema, right cardiac distension, general venous congestion and renal and suprarenal congestion. Typically, death appears to result from cardiac arrest but signs of generalized cardiovascular collapse combined with respiratory failure may be evident if death is not rapid. Studies by Barnes (1966), Southcott (1963), Cleland and Southcott (1965) and McNeill and Pope (1943a and b) further describe the symptoms of lethal and non-lethal stingings.

A second type of stinging attributed to the Cubomedusae is the so-called 'Irukandji' or Type A stinging as described

by Southcott (1959) and Barnes (1966). This produces minor local effects followed, after a considerable delay, by severe muscle spasms and vomiting with subsequent recovery. A small carybdeid medusa is reported to be the organism responsible (Barnes, 1964, 1966). Several other carybdeids are capable of inflicting relatively minor stingings (Cleland and Southcott, 1965).

PROBLEMS AND TECHNIQUES OF TOXIN ISOLATION

Investigation of cnidarian venoms is difficult because of the nature of the venom and its storage in the animal. Unlike venomous reptiles or molluscs which have either single or paired ducts and glands, cnidarian venom is associated with a multitude of microscopic nematocysts situated along the tentacles, and in some species, on the body surface as well. Thus, as pointed out by Barnes (1967) a sample intended to represent genuine cnidarian venom may theoretically be vitiated by

(a) entry of modifying substances from non-capsular sources

(b) loss of active constituents

(c) distortion of venom moiety ratios

(d) inclusion of contents of nematocysts not normally functioning as injectors

(e) inclusion of atypical contents of immature nematocysts.

It is important then that investigation of a species which causes injury by stinging should be made on the toxic material which is actually injected. This point can be appreciated when it is realized that many biologically active compounds have been identified in extracts of whole cnidarians. These include 5-hydroxytryptamine (5-HT), particularly concentrated in nematocyst-containing areas of tentacle (Russell, 1967; Welsh, 1960, 1961), tetramethylammonium hydroxide (tetramine), homarine and gamma-butyrylbetaine (Lane, 1968b; Welsh, 1961;

Welsh and Brock, 1958) and histamine-releasing substances (Russell, 1967; Welsh, 1961; Uvnäs, 1960). With the possible exception of 5-HT these compounds are unlikely to be venom components, thus their presence in tentacle extracts presents obvious difficulties.

Extracts containing high molecular weight, biologically active compounds have also been prepared and have been the subject of considerable investigation, although their function in the animal has not necessarily been shown to be that of venom. Martin (1960) and Farber and Lerke (1963) have obtained an extract of the anemone Rhodactis howesii which is toxic to toads and mice, stable over pH4.5 - 10.0, non-dialysable and inactivated by trypsin or by heating above 65°C. This extract also produces an immune response in rabbits. Both authors suggest that the material is either a protein or a small molecule closely associated with a protein but they have no information on its location within the anemone. Shapiro has isolated a basic protein of molecular weight 10,000 - 15,000 from an acetone extract of the anemone Condylactis gigantea. This material, which paralyses crustacea at a level of 1 µg/kg, appears to act by transforming action potentials in crustacean neurons into prolonged plateau potentials of up to several seconds duration (Shapiro and Lilleheil, 1969; Narahashi et al., 1969). The yield from a single anemone is sufficient to paralyse over 2000 kg of crayfish. All activity is lost after heating at 100°C for thirty minutes or after incubation at 23°C with 2% pronase for six hours. The toxin is non-dialysable and migrates as a single symmetrical peak on both gel filtration and ion exchange columns. On administration to crayfish it produces a spastic paralysis which is eventually replaced by flaccid paralysis and death. It is thought that the initial rigid stage is due to repetitive nerve firing whilst the flaccid paralysis results from prolongation of the nerve action

potential (Shapiro, 1968b). The action of the toxin is irreversible but is antagonized by calcium ions which may act by stabilizing the axonal membrane. Since the active material is apparently a single molecule and is extremely toxic to the crustacea which form the diet of the anemone it seems reasonable that it may represent Condylactis venom, however this has yet to be demonstrated directly.

All of the compounds just discussed have been isolated from tissue extracts of either whole animals or tentacles so that their relationship to true cnidarian venom is unknown. Some may be actual venom components but others are almost certainly present in general body tissues. Other workers have attempted to prepare toxin extracts which are free from contamination by general tissue components. This has been achieved by

(a) physical separation of the nematocysts from other tissue constituents

(b) a process analogous to snake milking

(c) preparation of some tentacle extracts.

(a) Physical separation. This method, which was originally described by Phillips (1956) for the anemone Metridium senile, involves maceration of the whole animal in 1M sucrose in sea water. The resultant homogenate is passed through graded sieves under suction and the material passing through the final mesh (0.147mm) consists of nematocysts, fine tissue debris and dissolved tissue constituents. Centrifugation and washing are then used to separate out the nematocysts. A modification of this method by Lane and Dodge (1958) for Physalia substitutes autolysis at 4°C for physical maceration as the initial step. Endean, Duchemin, McColm and Fraser (1969) use a similar method for isolation of nematocysts from Chironex fleckeri.

Discharge of isolated nematocysts is achieved by addition of distilled water or dilute acid or base (Phillips, 1956), homogenization and centrifugation (Lane and Dodge, 1958)

or grinding after successive washes in 0.9% NaCl (Endean et al., 1969).

(b) Tentacle 'milking'. This ingenious method is described by Barnes (1967). Prepared human amnion is spread across the mouth of a collecting chamber and lengths of tentacle are laid upon the surface. The tentacle is stretched slightly so that it adheres and electrically stimulated to produce nematocyst discharge. Only mature injectors penetrate the membrane to release toxin on the internal surface for collection. Chironex fleckeri, Chiropsalmus quadrigatus and Cyanea capillata all accept the amnion as a food object and discharge nematocysts. Obviously the method is tedious and the yield is small, but the extracts obtained are extremely valuable in assessing and comparing the activity of tissue extracts.

(c) Tentacle extracts. Extracts of tentacles from Chironex fleckeri have been prepared by grinding tentacular tissue in buffer solution and centrifugation. The supernatant has been purified by various chromatographic techniques and the biological properties of the various extracts have been compared with samples of 'milked' toxin. The use of tentacle extracts in this instance is considered to be valid because of the considerable body of evidence to suggest that these closely resemble Chironex venom. Animal responses to crude extracts and 'milked' toxin are identical and these correlate well with clinical descriptions of human stingings (Freeman and Turner, 1969a). Chromatographic analysis of both crude extracts and 'milked' toxin produces the same two active components in approximately the same proportions (Crone and Keen, 1970) and no evidence of low molecular weight biologically active compounds (5-HT, tetramine, etc.), which are present in the general tissues of many cnidarians, has been found (Freeman and Turner, 1969a).

Whilst the three techniques described above are undoubtedly superior to the preparation of 'whole animal' extracts they may not be completely representative of genuine cnidarian venom. Using Barnes' (1967) criteria the possible disadvantages of each method can be outlined. Modification of venom by entry of substances from non-capsular sources is a problem confined primarily to the use of tentacle extracts. To be valid, the response to such extracts must be correlated with either the response to a known pure toxin preparation or to an actual stinging by the species concerned. The loss of active constituents is a potential problem of all methods. The extreme lability of many of these toxins may cause distortion of component ratios even to the extent of removing a component(s) completely. Procedures involving nematocyst isolation are possibly more susceptible to such alteration because of the prolonged preparation compared with milking techniques or preparation of tentacle extracts. The method also assumes that permeability changes resulting in leakage of toxin do not occur. The inclusion of the contents of immature nematocysts would only be a modifying factor if there are actually toxic precursor compounds which differ from the mature venom. As this has not been demonstrated it seems equally possible that the venom, in its final form, is present in the cnidoblast cell and is included in the nematocyst at an early stage of its development.

Since each method of preparation has some disadvantages the main objective should be to define an extract which duplicates, in the experimental animal, the symptoms reported in cases of actual stingings. The only toxins prepared by these methods which have been investigated in detail are those from Physalia physalis and Chironex fleckeri.

PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF SOME CNIDARIAN TOXINS

Physalia toxin. Toxin extracted from Physalia nematocysts is lethal to all multicellular animals which have been tested. Human stingings are described by Russell (1966) and Cleland and Southcott (1965).

The crude toxin is non-dialysable and haemolytic. Its activity is markedly decreased by heating to 60°C for 15 minutes or by attempted extraction with acetone or ether. Chromatographic studies of crude lyophilized toxin have demonstrated the presence of glutamic acid and smaller quantities of cystine, cysteine, glycine, alanine, hydroxyproline, leucine, isoleucine and lysine (Lane and Dodge, 1958). Electrophoresis of the same extract separates four peptides one of which contains almost all the biological activity (Lane, 1961, 1968b).

Injection of the crude toxin into crustacea causes death by cardiac arrest in diastole (Lane, 1967). Mice respond to the toxin with increased activity, tremors and local irritation followed, after some minutes, by ataxia, decreased muscle tone leading to flaccid paralysis, respiratory depression, cyanosis, anoxic convulsions and death. Post mortem examination shows the lungs to be blanched, the heart is contracted and there is haemorrhagic oedema into the peritoneal cavity (Lane, 1960).

The cause of death is cardiovascular collapse (Hastings, Larsen and Lane, 1967; Garriott and Lane, 1969; Lane, 1967; Larsen and Lane, 1966). After intravenous administration of the toxin in rats, the first electrocardiographic abnormality is an inversion and/or reduction of the P wave accompanied by bradycardia. At low dose levels the Q-T interval is lengthened and at higher doses the slowed heart develops terminal ventricular patterns resembling paroxysmal tachycardia or fibrillation. T waves are reduced and QRS

complexes are notched or bifurcated. The hypotensive response observed after toxin administration is probably a secondary effect resulting from the direct cardiac actions of the toxin.

The ECG changes suggest interference with ventricular repolarization and atrioventricular conduction possibly by a generalized depolarization of cell membranes due to inhibition of active transport of ions. This concept is supported by the observation that serum potassium is significantly elevated and serum sodium depressed after toxin administration (Hastings et al., 1967). Lane (1968a) also reports that Physalia toxin inhibits ATP-ase in the gill epithelium of the land crab. This experiment lends further support to the concept that the toxin interferes with mechanisms of active transport.

Chironex fleckeri toxin. Until recently no detailed study of the mode of action of Chironex toxin had been published. Wiener (in Southcott and Kingston, 1959) noted that injection of tentacle extracts into mice and guinea pigs produced symptoms similar to those in human stingings. He also reported that these extracts were haemolytic and that toxicity was destroyed by boiling.

However, since 1968, studies of Chironex toxin have described several aspects of envenomation. 'Milked' toxin, extracts from washed nematocysts and tentacle extracts have all been used in these investigations and no significant differences in properties have been reported. The clinical picture of death from Chironex stingings correlates well with findings in experimental animals after intravenous injection of toxin extracts (Freeman and Turner, 1969a; Endean et al., 1969). The cause of death has been found to be respiratory arrest of central origin, associated with marked signs of cardiotoxicity (Freeman and Turner, 1969a, 1970a; Turner and Freeman, 1969). Properties of the

haemolysin have been investigated by Keen and Crone (1969a) and Endean and Henderson (1969) and the dermatonecrotic properties of tentacle extracts are described by Keen and Crone (1969b). It has been shown that Chironex toxin is antigenic and immune responses have been investigated by Baxter and Marr (1969), Baxter, Marr and Lane (1968), Keen and Crone (1969b), Keen (1970) and Endean et al. (1969).

Two lethal components have been separated from Chironex toxin preparations by Sephadex chromatography (Crone and Keen, 1969, 1970). The tentacle extract and the 'milked' toxin contained the same active components in approximately the same proportion. Both components (molecular weights 70,000 and 150,000) possess cardiotoxic properties, however only the lower MW fraction is haemolytic. The pharmacological properties of the two fractions have been investigated (Freeman and Turner, 1970a). Many of these findings are discussed in detail in the Results section of the thesis.

PART B OCTOPUS MACULOSUS TOXIN

INTRODUCTION

Octopus maculosus Hoyle (Hapalochlaena maculosa), the 'blue ringed octopus', is found in sheltered coastal waters of eastern and southern Australia, Japan, and some areas of the Indian Ocean (Cleland and Southcott, 1965). The species is small, maximum spread of the arms rarely exceeding 20 - 25cm, and can be recognized by the brown and yellow bands, superimposed with blue circles, which cover its body and arms. If the animal is disturbed or anoxic, the bands darken and the rings become a striking iridescent blue. O. maculosus has been responsible for three human fatalities and a number of cases of temporary paralysis; typically, the victims have been bitten while handling specimens found stranded in tidal rock pools.

The octopus belongs to the phylum Mollusca, a diverse group comprising approximately 80,000 known species of which 85 have either been implicated in human poisoning or are known to possess a toxin. The classification within the phylum is shown in Table 2; details of anatomy and

Table 2. Taxonomic classification with the phylum Mollusca.

MOLLUSCA

PELECYPODA GASTROPODA CEPHALOPODA SCAPHOPODA AMPHINEURA

development are described by Borradaile, Eastham, Potts and Saunders (1963). The majority of the dangerous species are confined to three classes, Gastropoda, Pelecypoda and Cephalopoda, and before discussing the effects of O. maculosus toxin in detail it is appropriate to discuss briefly some other toxic molluscs. This account is intended only as a summary and more detailed discussions may be found in Russell (1965), Halstead (1965), Cleland and Southcott

(1965) and Baslow (1969) and in specific references listed throughout the section.

DANGEROUS MEMBERS OF THE PHYLUM MOLLUSCA

Class: Pelecypoda (Lamellibranchiata). This class consists of molluscs with bilaterally symmetrical bodies that are laterally compressed and enclosed by shells which develop as two valves. Lamellibranchs are filter feeders with a highly developed gill system in which rows of cilia create water currents to carry food particles towards the mouth. The ingestion of toxic microorganisms by this method of feeding is responsible for the toxins, collectively described as paralytic shellfish poisons, which are attributed to several species. Over twenty species have been implicated in paralytic shellfish poisonings (Russell, 1965) including the mussel, Mytilus californianus, and the Alaskan butter clam, Saxidomas giganteus, from which saxitoxin is extracted. This toxin and its origin will be described later. The method of accumulation and concentration of these toxins is unknown.

Two other types of poisoning attributed to Lamellibranchs are described by Russell (1965). These are referred to as (a) gastrointestinal shellfish poisoning, caused by ingestion of a mollusc containing bacterial pathogens (see also Cleland and Southcott, 1965) and (b) allergic shellfish poisoning, characterized by an allergic response up to several hours after ingestion. Symptoms may range from skin rashes and headaches to respiratory distress, vomiting and, in rare cases, death. The nature of the sensitizing protein is unknown.

The giant clam, Tridacna gigas, has also been reported to be a harmful species, supposedly drowning unwary divers by rapidly closing its valves on a hand or foot. However, there is no real evidence that such an incident has occurred (Cleland and Southcott, 1965).

Class: Gastropoda. Molluscs in this group possess a head bearing tentacles and eyes. The shell is secreted in a single piece and is usually coiled. Gastropods may be carnivorous or may feed on algae.

The most dangerous group are the carnivorous Conidae or 'cone shells' which have developed a venom apparatus to facilitate capture of their prey. They are found in tropical and sub-tropical seas and are often common inhabitants of coral reefs. The Conidae are reported to be responsible for eleven deaths and many non-lethal stingings in humans (Kohn, 1963; Rice and Halstead, 1968). Species which are potentially dangerous to humans include C. geographus, C. striatus, C. tulipa, C. catus, and C. obscurus (Halstead, 1959; Kohn, Saunders and Wiener, 1960; Whyte and Endean, 1962; Endean and Rudkin, 1963; Russell, 1965; Endean, Izatt and McColm, 1967). Halstead (1959) and Kohn et al.(1960) also report C. textile as a dangerous species but Whyte and Endean (1962) were unable to produce toxic effects by intraperitoneal injection of venom duct extracts into mice or rats. Endean et al. (1967) suggest that only the piscivorous Conidae are capable of producing serious injury in man although some vermiferous species (C. quercinus and C. lividus) may cause local tissue damage at the site of injection.

The venom apparatus consists of a muscular venom bulb, a long, coiled venom duct and a chitinous radula tooth. The bulb acts as a storage and propulsive organ for the venom which is produced by the cells of the duct. Venom is transmitted to the prey by the radula tooth. The crude venom is viscous and granular and contains protein, lipid and polysaccharide (Endean et al.,1967). Chromatographic studies have also shown the presence of the quaternary ammonium compounds N-methylpyridinium, homarine and gamma-

butyrobetaine (Kohn, 1963; Endean et al., 1967). As no loss of venom activity is reported after dialysis, it must be assumed that the quaternary ammonium compounds are either contained within the venom granules or bound to some non-dialysable venom component. Heating of the venom alters, but does not remove, the toxicity.

Although the venoms of the many species of Conidae may differ, it is likely that those dangerous to man have a common mode of action. Injections of venom extracts from C. striatus or C. geographus into mice produce similar effects; the mice become ataxic, with respiratory difficulties and convulsions preceding respiratory failure and death. Both toxins produce a reversible decline of the muscle response to direct and indirect stimulation in the isolated phrenic nerve-diaphragm preparation. The block is not relieved by eserine (0.2 µg/ml) but, in the case of C. striatus toxin, it may be surmounted by increasing the intensity of both direct and indirect stimulation (Whyte and Endean, 1962; Endean et al., 1967). It is difficult to explain how an increase in the strength of nerve stimulation could break through the block unless this effectively produced a direct muscle stimulation by conduction of the current pulse along the exterior of the nerve.

The toxic manifestations suggest that the main action of the venom is an interference with the processes leading to contraction of skeletal muscle. This may be achieved by direct paralysis of the muscle (Endean et al., 1967), neuromuscular blockade (Kohn et al., 1960) or interference with peripheral nerve conduction (Russell, 1965). The ataxia and respiratory paralysis observed in mice after injection of Conus toxin could be explained by such an action, while the convulsions may result from the central

anoxia which would follow respiratory paralysis. Kohn (1963), however, does not exclude the possibility of a direct effect on the central nervous system.

It is interesting to note the similarity between the nerve or muscle block produced by Conus toxin and that described by Kuriaki and Wada (1957) for the puffer fish toxin, tetrodotoxin. Application of tetrodotoxin to an isolated rat phrenic nerve-diaphragm preparation produced a depression of the muscular response to both direct and indirect stimulation which was unaffected by eserine. However, even after complete paralysis, direct stimulation at greater intensity sometimes produced a contraction. The venom of the Conidae may be related to tetrodotoxin but modified by the presence of active protein or quaternary ammonium compounds.

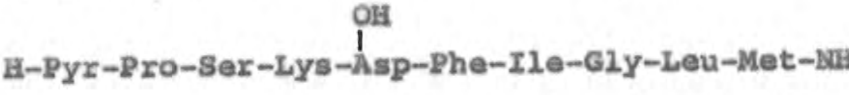
Several other gastropods are known to be venomous or poisonous (Russell, 1965). These include Neptunea arthritica (Asano and Itoh, 1960) and Neptunea antiqua (Fänge, 1960). Tetramine is reported to be the major component of the salivary poisons of both species. Another quaternary ammonium compound, urocanylcholine (Murexine) is found in several gastropods (Erspamer and Glässer, 1957; Whittaker, 1960). Murexine is a ganglion stimulant and a depolarizing neuromuscular blocking agent about one-fifth as potent as succinylcholine.

Class: Cephalopoda. This class includes the Octopoda and the Decapoda (squid), bilaterally symmetrical molluscs which possess a well-developed head surrounded by mobile and prehensile tentacles. The nervous system is greatly centralized and the eyes are large and complex.

Human injuries may be caused by mechanical contact with the arms of a large species or by a bite from the beak-like jaws and administration of a salivary venom.

Several incidents involving octopus bites in humans are described by Flecker and Cotton (1955), McMichael (1955, 1964), Hopkins (1964) and Cleland and Southcott (1965).

The venom is produced and secreted by the large posterior salivary glands situated in the dorsal region of the body. Single ducts from each gland combine to form a common duct which opens into the buccal cavity at the base of the radula. Active compounds which have been identified in the posterior salivary glands of the Octopoda include amines, peptides and proteinaceous materials. An indication of the range of compounds is given in Table 3. Eledoisin (Moschatin) is an endecapeptide with the amino acid sequence



It is a powerful hypotensive agent and exhibits strong stimulant actions on extravascular smooth muscle. Cephalotoxin produces paralysis, cardiac arrest and inhibition of respiration in some crustaceans. Experiments by Ghiretti (1960) have shown that cephalotoxin is heat labile and inactivated by trypsin, and preliminary studies indicate that it may be a glycoprotein. O. maculosus toxin (maculotoxin) affects nerve and muscle activity in the isolated nerve-muscle preparation (Simon, Cairncross, Satchell, Gay and Edwards, 1964; Trethewie, 1965; Freeman and Turner, 1970b). In the intact animal the toxin produces generalized skeletal muscle paralysis, hypotension and, in sufficiently large doses, death due to respiratory paralysis. The effects of maculotoxin are discussed in detail in the Results section of the thesis.

The experiments of Freeman and Turner (1970b) have shown that maculotoxin possesses several properties in common with both the puffer fish poison, tetrodotoxin, and

Table 3. Compounds isolated from posterior salivary glands of Octopoda.

Compound	Species	References
Amines		
5-OHtryptamine	O.vulgaris, O.bimaculatus	Erspamer,1954; Hartman et al.,1960.
histamine	O.macropus, O.apollyon, O.bimaculatus	Hartman et al.,1960; Russell,1967.
adrenaline	O.apollyon	Russell,1967;
noradrenaline	O.apollyon	Hartman et al.,1960.
tyramine	O.vulgaris, O.macropus, Hapalochlaena maculosa	Erspamer and Boretti, 1951; Russell,1967; Simon et al.,1964.
dopamine	O.apollyon	Hartman et al.,1960.
octopamine (L-p-OHphenyl-ethanolamine)	O.vulgaris, H.maculosa	Erspamer,1952; Simon et al.,1964.
Amino acids		
tyrosine	O.apollyon, O.bimaculatus	Hartman et al.,1960.
histidine	O.apollyon, O.bimaculatus	Hartman et al.,1960.
Other compounds		
maculotoxin	H.maculosa	Freeman and Turner, 1970b.
eledoisin	Eledone moschata, E.aldrovandi	Erspamer,1949.
cephalotoxin	O.vulgaris, O.macropus	Ghiretti,1959,1960.
amino acid decarboxylases	O.bimaculatus	Hartman et al.,1960.
flavinic enzymes	O.apollyon	Hartman et al.,1960.
amine oxidases	O.apollyon	Hartman et al.,1960.
proteolytic enzymes	O.vulgaris	Ghiretti,1960.
hyaluronidase	O.vulgaris	Ghiretti,1960.

the clam poison, saxitoxin. The similarities and differences are best described by summarizing the actions of the three toxins.

A COMPARISON OF MACULOTOXIN WITH TETRODOTOXIN AND SAXITOXIN

SOURCES OF THE TOXINS

Tetrodotoxin (TTX) is found in the ovaries, liver, skin and intestines of several species of Tetrodont and other fishes. TTX is an amino perhydroquinazoline derivative with the empirical formula $C_{11}H_{17}O_8N_3$. The structural formula as given by Goto et al.(1963) is shown in figure 2. The history of tetrodotoxin merges into folklore and it is referred to in Chinese medicine as early as 200 B.C. Even today a considerable number of fatalities result from the ingestion of poisonous species; in Japan for instance, approximately 100 deaths occur each year (Russell, 1967). TTX is also found in the eggs and skin of the California newt, Taricha torosa. The newt toxin, first reported by Twitty and Elliott in 1934 and named tarichatoxin, has since been found to be identical to TTX. Methods of preparation are described by Yokoo (1950), Goto et al. (1963) and Goto, Kishi, Takahashi and Hirata (1965).

Saxitoxin (STX) is obtained from several species of mollusc including the Alaskan butter clam, Saxidomas giganteus and the mussel, Mytilus californianus, but the mollusc is not the primary toxin source. Studies by Sommer and Meyer (1937) and Sommer, Whedon, Kofoid and Stohler (1937) suggested that the toxin was transferred to the shellfish from microorganisms in the sea water, and these and subsequent investigations suggested the dinoflagellate, Gonyaulax catenella, as the likely toxin source for

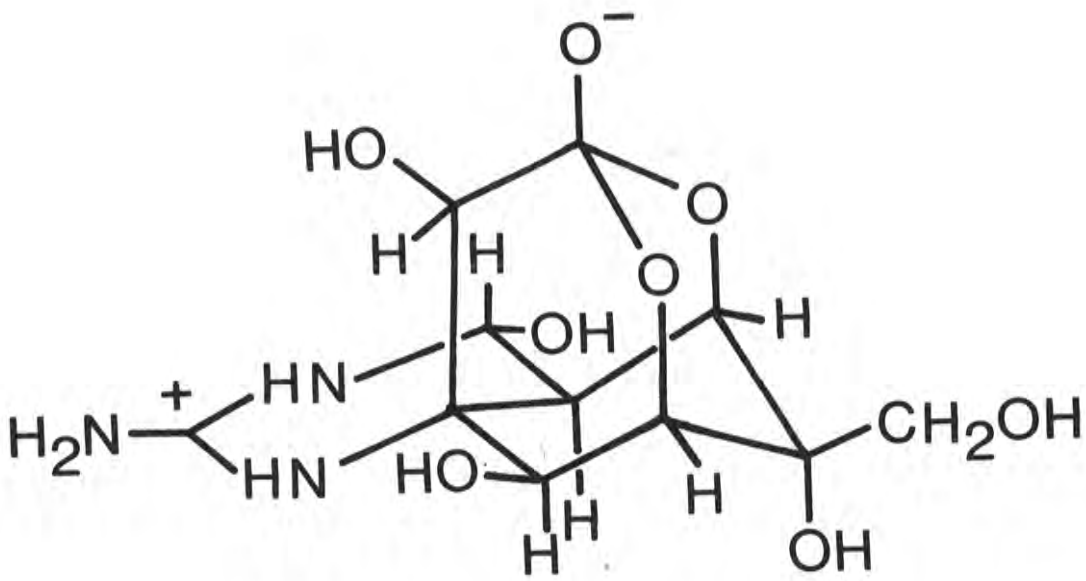


Figure 2: Structure of tetrodotoxin.

M. californianus. The production of a toxin, indistinguishable from saxitoxin, from axenic cultures of G. catenella has confirmed this suggestion (Burke, Marchisotto, McLaughlin and Provasoli, 1960). The primary source of the clam toxin has still to be identified. Saxitoxin is a basic compound, the empirical formula of the dihydrochloride salt is $C_{10}H_{17}N_7O_4.2HCl$. The structural formula has not been completely established. Techniques of extraction and purification of STX are described by Schantz et al. (1957) and Schantz (1960).

Maculotoxin (MTX) is derived from extracts of the posterior salivary glands of O. maculosus. No other species of cephalopod has been reported to possess a similar toxin (Simon et al., 1964). A detailed chemical analysis has not been made, but initial studies indicate that maculotoxin has a molecular weight of the same order as TTX and STX and behaves similarly on cation exchange chromatography on Amberlite IRC-50 (Goto et al., 1965; Schantz et al., 1957; Freeman and Turner, unpublished). Biological properties of the three toxins are also very similar.

It is fascinating that related toxins are present in organisms as vastly different as protozoans, molluscs, fishes and amphibians and even more intriguing that relatively few members of any group possess the toxin. What combinations of evolutionary syntheses resulted in this strange distribution and what possible role the toxin plays in the life of the dinoflagellate are perplexing questions which, fortunately, are irrelevant to this survey.

BIOLOGICAL ACTIONS OF THE TOXINS

Saxitoxin, tetrodotoxin and maculotoxin are among the most potent animal toxins. They are more than 100,000 times as potent as cocaine in blocking axonal conduction, and a dose of less than 200ng is sufficient to kill a mouse.

Only symptomatic treatment is available for human victims, and mortality is high, approximately 60 per cent for tetrodotoxin poisonings (Russell, 1967). Incidents involving human poisonings are described by Sommer and Meyer (1937), Halstead (1964) and Cleland and Southcott (1965). An excellent review of the general properties of tetrodotoxin and saxitoxin has been compiled by Kao (1966).

Mouse toxicity. The toxins all produce similar effects on intraperitoneal or intravenous injection. Mice become agitated and ataxic, and the limbs splay out and become paralyzed. General muscular weakness and laboured respirations are followed by convulsions and respiratory arrest. The heart frequently continues to beat for some time after respirations cease.

The dose-mortality curve is extremely steep, making calculation of an LD_{50} difficult. However, if standardized techniques are used such data may be useful in the identification of these toxins (Kao, 1966). Graphs prepared from dose versus time to death data for saxitoxin possess a significantly different slope from those prepared for tetrodotoxin or maculotoxin (Konosu, Inoue, Noguchi and Hashimoto, 1968; Freeman and Turner, 1970b).

Effects on the anaesthetized animal. TTX, STX and MTX all produce a hypotensive response upon administration to the anaesthetized animal. In each case the fall in blood pressure appears to result mainly from generalized vasodilatation as little or no change in pulse pressure is observed even after doses which reduce arterial pressure by 50 per cent. Similarly, heart rate was unaffected by TTX or STX (Kao, 1967) although comparable doses of maculotoxin reduced the heart rate by 15 - 20 per cent (Freeman and Turner, 1970b). Administration of catecholamines restored the blood pressure to control levels in all cases.

The exact cause of the hypotension is not clear. Initially, this was attributed to depression of the medullary vasomotor centre, but cross-perfusion experiments by Kao, Suzuki, Kleinhaus and Siegman (1967), in which the head of an anaesthetized dog or cat could be perfused separately from the body, showed that neither tetrodotoxin nor saxitoxin produced a hypotensive response if perfused in the head alone. Hypotension is probably produced by block of peripheral vasomotor nerves and/or the spinal vasomotor areas.

All three toxins decrease respiratory amplitude and rate. Rate changes are typically less than 20 per cent and amplitude changes are dependant on the dose of toxin administered. Recordings of phrenic nerve and diaphragmatic activity show that the phrenic nerve continues to conduct bursts of impulses after diaphragmatic activity has ceased (Cheng, Ling and Wang, 1968; Freeman and Turner, 1970b). Saxitoxin differs from the other two toxins in that small doses may produce respiratory depression and general skeletal muscle weakness without producing hypotension. Doses of either TTX or MTX which produce minimal respiratory depression also cause a fall in blood pressure. These differences may reflect a greater selectivity of action on somatic motor nerves by saxitoxin.

Effects on the central nervous system. The cross-perfusion experiments of Kao et al. (1967) showed that the major effects of tetrodotoxin and saxitoxin on the cardiovascular and respiratory systems are peripheral. However, it is likely that compounds which possess such potent nerve blocking activity would have some central effects if they are able to cross the blood-brain barrier. The centrally-mediated emetic and hypothermic properties of TTX

demonstrate that at least some areas of the brain are accessible. Saxitoxin does not act as an emetic or a hypothermic agent (Kao, 1967) and it is not known if maculotoxin possesses these properties, although reports that victims have vomited after bites by O. maculosus suggest a possible emetic effect.

Effects on nerve and muscle. The three toxins all produce reversible conduction block in isolated nerve (Kao and Nishiyama, 1965; Simon et al., 1964; Freeman and Turner, 1970b). The studies on MTX also showed a decrease in conduction velocity in rat and toad sciatic nerve, with consequent broadening of the compound action potential due to sequential changes in conduction velocity in different fibre groups. These changes are not reported for nerve blockade with TTX or STX.

Application of any of the toxins to a nerve-muscle preparation results in a decrease in the amplitude of the nerve-evoked muscular response. Large doses or prolonged treatment also renders the muscle unresponsive to direct stimulation (Kuriaki and Wada, 1957; Kao, 1966; Freeman and Turner, 1970b). Compounds such as d-tubocurarine, decamethonium, acetylcholine and anticholinesterases have no effect on the response to the toxin. Both nerve and muscle blocks occur without depolarization. Investigation of the blockade in a nerve-muscle preparation by intracellular recording reveals a stepwise decrease in the size of the end-plate potential with no effect on the miniature end-plate potential (m.e.p.p.) (Kao and Nishiyama, 1965; Katz and Miledi, 1966; Dulhunty and Gage, 1970). This would suggest that the block occurs in the motor axon and not in the end-plate receptors. Saxitoxin may cause a reduction in m.e.p.p. amplitude after long exposure (Kao, 1966). None of the toxins possess any

anticholinesterase activity (Russell, 1967; H.D. Crone, personal communication).

Mechanism of action of the toxins. All the effects of the toxins may probably be explained in terms of their depressant action on nerve and muscle action potentials. Slight differences in properties may be explained by structural differences in the molecules resulting in differential selectivity of action. The action of both tetrodotoxin and saxitoxin is to prevent the early, transient increase in ionic permeability which allows the downhill inward movement of sodium ions during the action potential (Kao and Nishiyama, 1965; Kao, 1967). The outward potassium current which develops more slowly is totally unaffected. Experiments by Moore, Blaustein, Anderson and Narahashi (1967) in which Li^+ was substituted for Na^+ in the external medium have shown that the initial decrease in ion conductance is not Na^+-specific. Membranes which depend on Ca^{++} movements for excitation, however, are not affected by tetrodotoxin or saxitoxin. The effect of maculotoxin on ion fluxes has yet to be determined.

Tetrodotoxin is ineffective if applied intracellularly (Narahashi, Anderson and Moore, 1967) thus indicating that it acts on the external surface of the excitable membrane, possibly by preventing the transient openings of the early channels. The presence of guanidinium groups in TTX and STX (Kao, 1966) and the ability of guanidine to pass through the membrane sodium channels has led to the suggestion that the toxins act by effectively 'plugging' these channels. However, experiments by Narahashi, Moore and Poston (1966) with tetrodotoxin derivatives have shown that the presence of a guanidinium group is not necessarily sufficient for the

molecule to be potent. It is possible that the guanidinium group requires a particular spatial relationship with secondary binding groups for such a 'plug' to be effective.

Positive diacetyl and Fearon's tests suggest that maculotoxin may also contain a guanidinium group, but more specific tests are necessary to confirm this (M.W. Jarvis, personal communication).

Toxin specificity. The intriguing distribution of these toxins throughout various animal groups has already been mentioned. A point of further interest is that some species are resistant to the effects of one or other of the toxins. The newt, Taricha torosa, is unaffected by doses of tetrodotoxin which are more than 7000 times the frog lethal dose (Kao and Fuhrman, 1967). Both tetrodont and Taricha nerves are extremely resistant to block by TTX; desheathed Taricha nerve requires tetrodotoxin concentrations greater than 300 μM (100 times the blocking dose of saxitoxin) to produce block. The reason for such resistance is not known; action potentials in both nerves are 'Na^+ spikes' and electron microscopy of newt and frog nerve has revealed no significant structural differences. The existence of species resistance to maculotoxin has not yet been investigated.

Status of maculotoxin. From the preceding account it is obvious that maculotoxin shares many properties with tetrodotoxin and saxitoxin. Further studies of the chemistry and physiological effects of maculotoxin are necessary to determine how far this similarity extends and to investigate any differences which may be found.

METHODS

COLLECTION OF SPECIMENS

Live specimens of Chironex fleckeri were caught in North Queensland waters in mid-summer by Dr. J.H. Barnes and maintained in sea water containers at approximately 8°C for 3 to 24 hours. They were then carefully lifted from the sea water by grasping the apex of the umbrella and the tentacles were cut off at each pedalium and allowed to fall into a container of liquid nitrogen. The tentacles were then freighted to the laboratory in this container. Precooling of the animals before freezing appeared to reduce nematocyst discharge to a negligible amount because nematocysts isolated from the tentacles were largely undischarged (Fig. 3).

Live specimens of Octopus maculosus Hoyle, collected in Victorian coastal waters, were obtained from members of the public. Initially, the animals were maintained in an aquarium until required, and fed daily on small crabs. However, on several occasions they died when the sea water was changed, apparently due to the presence of a pollutant. Thereafter, the animals were killed immediately by incision of the central ganglion and the posterior salivary glands were removed and stored in liquid nitrogen.

PREPARATION OF TOXINS

CHIRONEX FLECKERI TOXIN

Crude toxin extract. Thawed tentacle was minced in a 7%

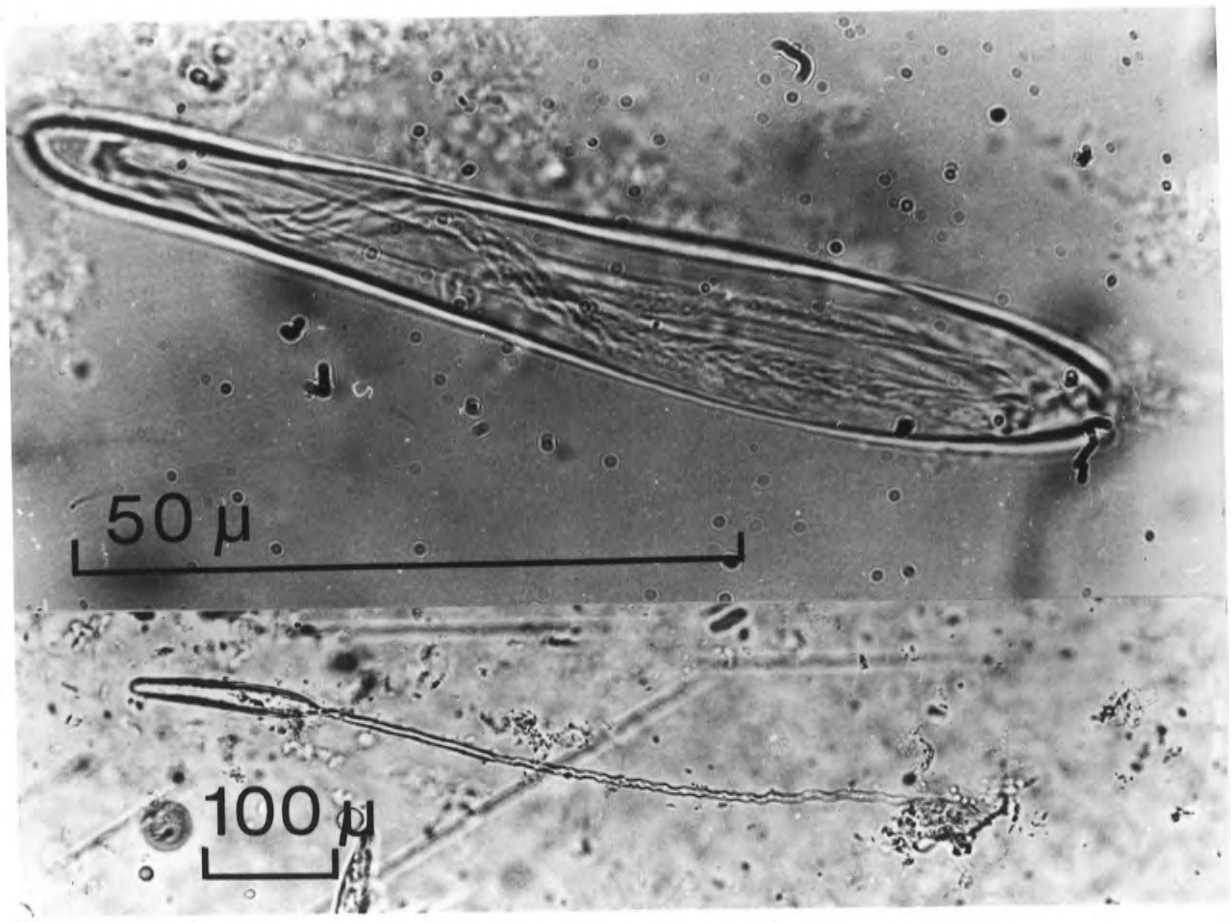


Figure 3: Nematocysts isolated from frozen Chironex tentacle. These are microbasic mastigophores; tentacles contain several other types (Barnes, 1967). The upper picture shows the undischarged capsule; most were in this state in the frozen tentacle. The lower picture shows a discharged capsule with the long lash intact.

sucrose solution. Robson (1953) found that nematocyst discharge in Corynactis was reduced by sucrose. The resulting suspension was strained through a fine, stainless steel mesh to remove coarse particulate matter and then centrifuged for 5 minutes to precipitate the nematocysts. All operations were carried out at 5°C. It was hoped that the toxic material would be retained within the undischarged nematocysts, however most toxicity was found in the supernatant. Grinding the precipitated matter in a glass homogenizer or sonic disintegration did not increase the toxicity of this fraction. Possibly the freezing and thawing caused changes in nematocyst permeability resulting in leakage of toxins although the existence of toxin outside the mature nematocyst (perhaps in cnidoblast cells at an early stage of nematocyst development) cannot be excluded.

The finding that the toxic material was present in the supernatant rather than in the precipitated nematocysts after this procedure meant that further nematocyst isolation was of little use. Consequently, the Chironex toxin fractions used in these experiments have been derived predominantly from whole tentacle extracts. This approach is considered to be valid for several reasons:

(a) the pharmacological properties of crude tentacle extracts, Sephadex G-75 eluates and amnion 'milked' toxin were identical in all experiments. In addition, the signs which are described in clinical reports of human Chironex stingings are very similar to those observed in anaesthetized animals after injection of tentacle extracts.

(b) no biologically active, low molecular weight compounds, which could originate from general tentacular

tissues, were found. If these were present in the crude extract they would be removed by Sephadex G-75 chromatography or dialysis. Neither of these procedures produced an extract with properties different from the crude extract.

(c) the toxin is extremely potent in its action; 0.1 µl of a typical tentacle extract was sufficient to kill a 20g mouse. This meant that any contaminating substances would be present in negligible quantities after the dilution required to reach experimental doses (usually less than 20 'mouse units'). Modification of the action of the toxin by other tissue constituents was thus unlikely.

(d) cation exchange Sephadex chromatography of tentacle extracts or 'milked' toxin produced the same two active components in approximately the same proportions.

Toxin fractions prepared by Sephadex chromatography.

For the initial series of experiments, some tentacle extracts were partially purified by exclusion chromatography on Sephadex G-75 at 5°C. The eluting buffer was 10mM Tris - (hydroxymethyl) aminomethane (Tris) maleate, pH 6.3 (Lane, 1967). The lethality of the eluted fractions was tested by injection into the lateral tail veins of 20g mice. Peak lethality appeared in fractions eluted from the column a few ml after the void volume (corresponding to a molecular weight greater than bovine serum albumin).

Investigations by Crone and Keen (1969) in these laboratories demonstrated that the crude toxin could be separated, by exclusion chromatography on Sephadex G-200, into two lethal components with differing pharmacological properties. For later work on the properties of the two

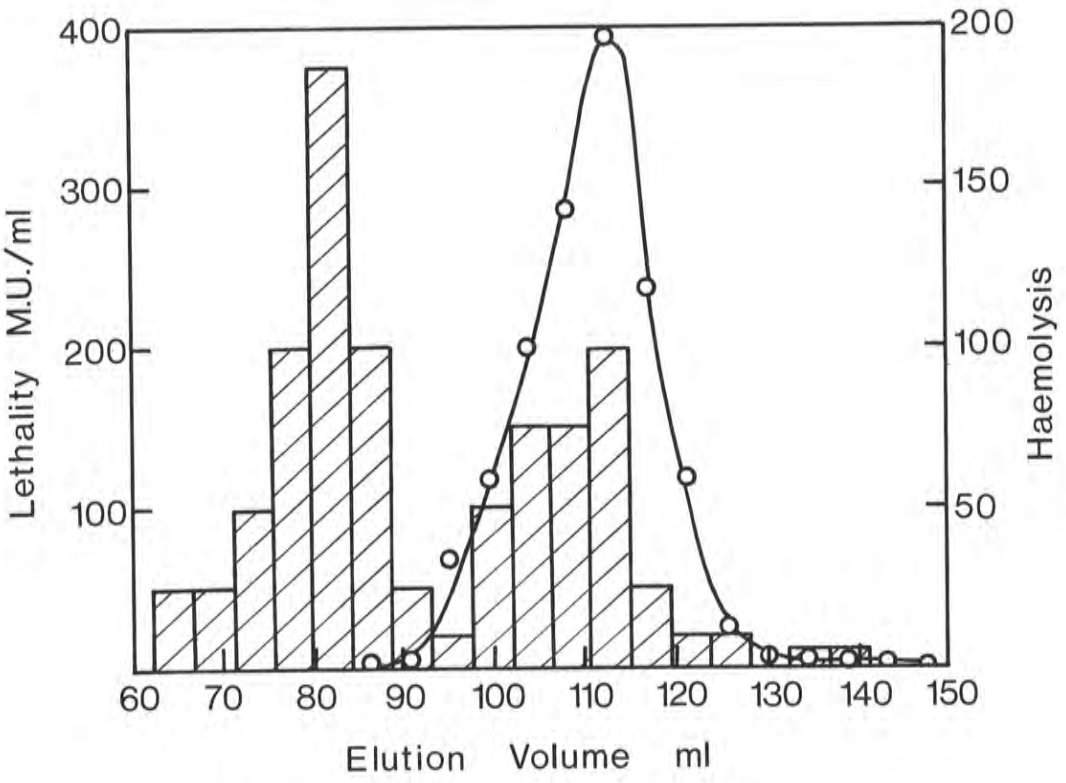


Figure 4: Separation of cardiotoxic and haemolytic fractions by Sephadex G-200 column chromatography. The void volume as defined by a dextran blue marker was 65 ml. ▨ Lethality: 0—0 Haemolytic activity.

fractions the following method of toxin preparation was used. A crude extract was prepared by grinding the tentacle in a mortar with liquid nitrogen and adding saline buffer in the proportion of 1 ml per 400mg tentacle. After centrifugation at 5000g for 15 minutes a 3 ml sample of supernatant was removed and applied to a Sephadex G-200 column. The eluting buffer was 5mM Tris-HCl, 150mM NaCl, pH 8.0. The flow rate, at an operating pressure of 14 cm H_2O, was approximately 5 ml per hour. 4 - 5 ml fractions were collected. All preparation and chromatography was performed at 5°C and column fractions were stored at -20°C.

Figure 4 shows that two well-defined lethal fractions separated on Sephadex G-200. Both fractions possessed cardiotoxic properties, however only the 70,000 MW fraction was haemolytic. For pharmacological investigations only the peaks of these fractions, corresponding to elution volumes from 75 to 90 ml and 105 - 115 ml, were used. The area under the lethality curve for the two fractions consistently suggested that the cardiotoxic fraction (MW 150,000) was the major component. This may, however, be due to the instability of the toxins at 5°C since the haemolysin took longer than the cardiotoxin to pass through the column.

'Milked' toxin. This was obtained from Dr. J.H. Barnes and was prepared as described earlier and in Barnes (1967).

MACULOTOXIN

Glands from different animals contained widely differing amounts of toxin per mg gland tissue; in particular

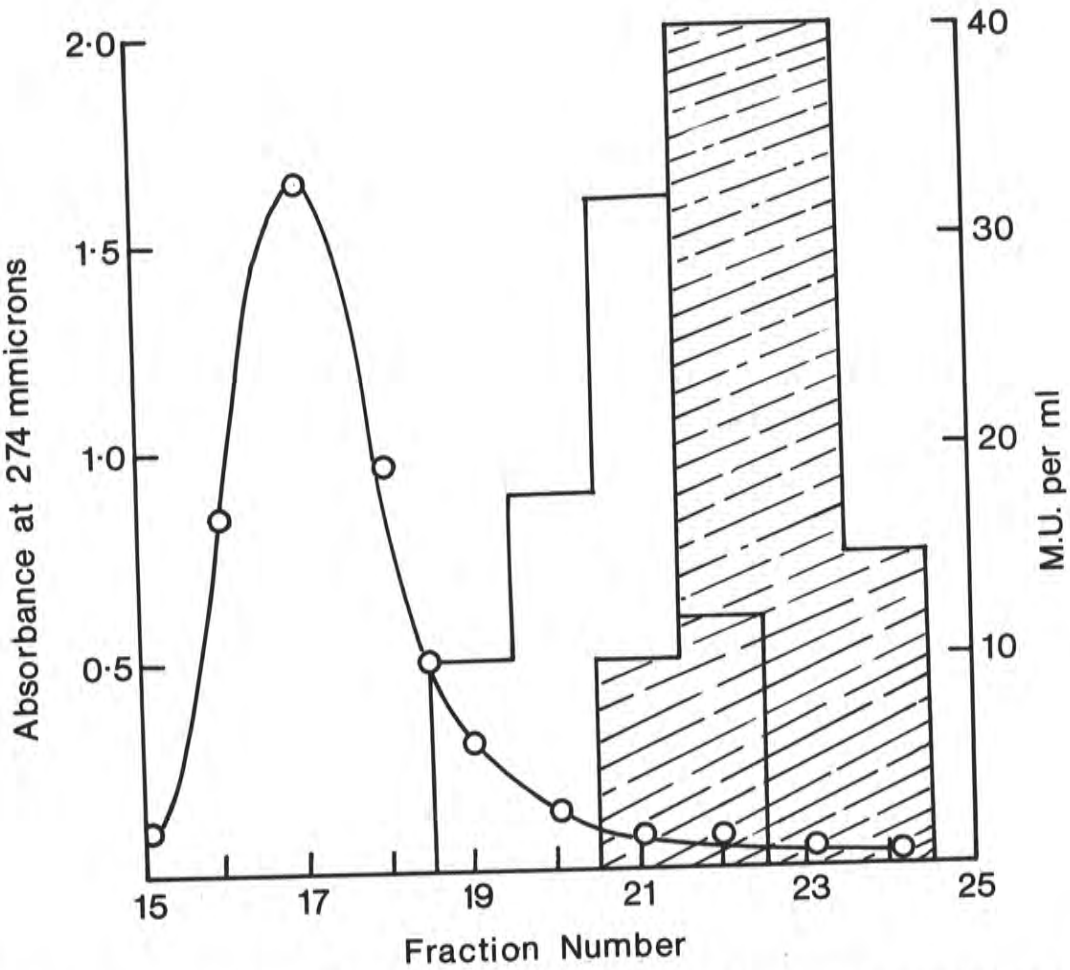


Figure 5: Separation of maculotoxin from an aqueous extract of O. maculosus salivary gland by Sephadex G-25 column chromatography. Maculotoxin (hatched area) is well separated from an ultraviolet absorbing contaminant (0 — 0), Saxitoxin (unhatched area) was run through the column on the following day under identical conditions. 5 ml fractions are numbered from the centre of the blue dextran void volume marker. Void volume was 85 ml; flow rate 40 ml per hour.

it was noted that animals in poor condition frequently contained virtually no toxin. Consequently the present study was carried out on an extract made from the pooled glands of 11 animals. 3.2g tissue was homogenized in 30 ml distilled water and the debris centrifuged down. The water soluble toxin was partially purified by Sephadex G-25 column chromatography, the eluting buffer was 5mM Tris - HCl, 150mM NaCl, pH 8.0. Lethal activity was eluted from the column in a discrete peak which contained approximately 70% of the lethality of the original extract, and consequently must represent the major or only lethal component.

It was possible by this procedure to separate the lethal fraction, which does not absorb in the ultraviolet region, from a contaminant which absorbs at 274 mμ (Simon et al., 1964). The study by Simon et al. was also complicated by the fact that acetone extracts of the glands were used as starting material. It has now been shown that the toxin is only partially soluble in acetone.

Figure 5 illustrates the separation of maculotoxin from the ultraviolet-absorbing material by Sephadex G-25 chromatography. The elution volume of saxitoxin is also shown for comparison.

ASSAY OF SAMPLES

Lethality. The intravenous LD_{50} for Chironex toxin samples was determined in 25g mice, using the tables of Weil (1952). Because of the instability of the toxin, however, the MLD was determined daily immediately prior to use. The minimum dose which was lethal to a group of 4 - 5 mice within ten

minutes was taken as the 'mouse unit' (M.U.). This unit is used in all experiments and offers some means of assessing toxin activity on various preparations.

The lethality of maculotoxin in 23 - 25g mice was determined by injection of 0.1 ml of material with a Hamilton 100 microlitre syringe into the lateral tail vein, or by intraperitoneal injection of the toxin diluted to 1 ml with distilled water. The minimum dose which was lethal within ten minutes was defined as 1 M.U. It was found that the intravenous mouse unit was comparable to the intraperitoneal mouse unit, as defined by workers with saxitoxin and tetrodotoxin (Schantz, 1960; Kao, 1966) (See also Results section).

Haemolysis. The method described by Crone and Keen (1969) was used to determine the haemolytic activity of Chironex toxin. This is based on observation of the time to complete haemolysis at 25°C, the end point being judged by eye. Toxin samples of 0.1 ml were added to 1 ml of rabbit red cell suspension (approx. 3×10^7 cells per ml) at pH 7.4 and the observed time to haemolysis was converted to relative dilution by means of a calibration curve. The reciprocal of this value multiplied by 1000 was used as an arbitrary estimate of the haemolytic activity.

WHOLE ANIMAL EXPERIMENTAL TECHNIQUES

The effects of the toxins on various physiological parameters were tested using New Zealand rabbits (2 - 3kg) or Wistar rats (250 - 300g). The rabbits were anaesthetized by intravenous injection of 20% urethane into the marginal ear vein or, in later experiments, by 20% urethane

supplemented with chloralose (Korner, Uther and White, 1968). Rats were anaesthetized with 40 - 50mg/kg pentobarbital injected intraperitoneally, supplemented if necessary by small doses administered through a hypodermic needle taped into a lateral tail vein.

Arterial pressure was measured by catheterization of the right carotid artery in rats and rabbits or, in some rabbit experiments, the right femoral artery. Right atrial pressure was recorded in both animals by passing a polythene cannula down the left jugular vein to the level of the right atrium. The pressures were monitored with Statham P23Db (arterial) and P23BB (right atrial) pressure transducers connected to a Beckman type R dynograph recorder.

Lead 1 electrocardiographs were recorded both directly and via a cardiotachometer which gave a direct measure of heart rate, the counter being triggered from the R wave. Needle electrodes were used.

Respiratory rate and depth were recorded with a colloidal carbon-in-rubber pneumograph placed just below the xiphisternum. The pneumograph formed a variable resistance arm in a Wheatstone bridge circuit, the output of which was displayed on the pen recorder.

Plasma electrolytes were determined in heparinized samples with an EEL flame photometer.

Several experiments involved the monitoring of electrical activity in the phrenic nerve and the diaphragm of anaesthetized rabbits. The phrenic nerve was exposed in the neck and placed upon a pair of platinum electrodes; a concentric needle electrode recorded diaphragmatic activity. Both signals were amplified with Tektronix Type 122

preamplifiers (filtered below 8 Hz and above 1 kHz) and displayed on a Tektronix Type 502 dual beam oscilloscope. The display was photographed on moving film at 15 metres/min with a Dumont Oscillograph-Record camera.

A series of experiments with the Chironex toxins isolated by Sephadex G-200 chromatography involved the investigation of baroreceptor reflexes in the rat after acute section of the aortic and carotid baro- and chemoreceptor nerves. By careful dissection of the cervical region with the aid of a magnifying loupe, the superior laryngeal, recurrent laryngeal, aortic depressor and vagus nerves and the sympathetic trunk were sectioned, together with the nerves arising in the carotid bifurcation (Krieger and Marseillan, 1963).

The effect of crude Chironex tentacle extract on the release of vasoactive substances from the skin was tested using a technique similar to that of Rocha e Silva and Rosenthal (1961). An air pocket was formed under the dorsal skin of an anaesthetized rat, the animal was hung upside down by its four paws and successive 5 ml washings of bathing solution were introduced into the air pocket. Toxin, equivalent to approximately five lethal doses if given intravenously, was injected intradermally into the pouch after the first control wash. The samples were assayed on an isolated guinea pig ileum preparation against a standard histamine dose-response curve. The animal was finally killed by injection of the toxin into the tail vein.

ISOLATED ORGAN PREPARATIONS

Phrenic nerve-diaphragm preparation (Bülbring, 1946). The rat hemidiaphragm was mounted on a perspex holder in a 20 ml organ bath and the nerve placed over two platinum stimulating electrodes. Stimulation of the preparation was by supramaximal pulses of 0.15 msec duration at a rate of 12 per minute.

Isometric twitch tension was measured with a Statham UC-2 transducing cell and recorded on a Beckman dynograph recorder. Bath temperature was 29 - 30°C.

Denervated rat diaphragm. 200 - 250g rats were anaesthetized with 40 - 50mg/kg pentobarbital and the left hemidiaphragm was denervated by evulsion of the phrenic nerve in the neck. After postoperative maintenance for 8 - 21 days, to allow the nerve to degenerate, the animal was killed by a blow on the head, exsanguinated, and the denervated diaphragm mounted in a 20 ml organ bath. Isometric tension developed by the muscle in response to drugs or electrical stimulation was recorded with a Statham UC-2 transducing cell connected to a Beckman dynograph recorder.

Sciatic nerve. The effect of maculotoxin on toad and rat sciatic nerves was investigated. Toad nerve was desheathed with a pair of fine needles prior to recording; rat nerve proved difficult to desheath so the relatively fine peroneal branch was used. The nerve was stimulated with supramaximal pulses of 0.1 msec duration and the action potentials recorded with platinum electrodes placed 1.5 - 2.0cm along the nerve. The signal was amplified with a Tektronix 122 preamplifier and displayed on a Tektronix 564 oscilloscope.

Guinea pig ileum. 4 - 5cm segments from the distal section of the ileum were mounted in organ baths. Changes in tension resulting from addition of drugs or toxin were measured semi-isometrically by a strain gauge transducer connected to a pen recorder. Bath temperature was maintained at 29 - 30°C to reduce spontaneous activity.

Guinea pig trachea (Jamieson, 1962). A 3cm segment of trachea was cannulated at both ends and mounted in a temperature controlled organ bath. The upper cannula was connected to a vertical, graduated capillary tube (1mm bore), the trachea was filled with bathing solution and the level adjusted so that it

could be read on the graduated scale. Test solutions were added to the bath and the changes in fluid level in the capillary tube (representing constriction or relaxation of the trachea) were noted 2.5 minutes after addition. Responses were measured against a histamine dose-response curve. Bath temperatures of 29°C and 37°C were used.

Histamine release from mast cells. This was measured by the technique of Rothschild (1962). Peritoneal fluid cells from 300g rats were collected by flushing the peritoneal cavity with 10 ml phosphate buffer, pH 7.3. After centrifugation the cells were resuspended in 2 ml buffer. 1 ml of the suspension was added to 2 ml buffered Chironex tentacle extract (approx. 2000 M.U.) and incubated for 20 minutes at 30°C. A blank contained cells and buffer only. After incubation, the cells were washed twice in physiological saline, lysed with 0.1 M HCl at 70°C for 20 minutes and neutralized before assay of residual histamine on guinea pig ileum. Histamine content of the control was taken as 100% and the content of the test sample expressed as a percentage of this. The specificity of the histamine-induced contraction was determined by blocking the response with 6.8×10^{-8}M diphenhydramine.

Perfused guinea pig heart. Hearts from 300 - 450g guinea pigs were used. The animals were anaesthetized with a 50% CO_2/50% O_2 mixture (Satchell, Freeman and Edwards, 1968) or ether; the hearts were rapidly removed, the aorta cannulated and the preparation mounted in the perfusion apparatus (Satchell, 1967). The time from opening of the thoracic cavity to the beginning of perfusion was 3 - 4 min. Twin reservoir systems enabled rapid changes of fluid composition when required. The perfusion pressure was 65cm water.

Heart rate and isotonic contraction were measured with a differential transformer. This consisted of three 120 ohm coils with a 3 volt 400 Hz signal supplied to the centre coil.

Vertical movement of a ferrite core centrally produced an output across the outer coils. For use, the coils were mounted in a Perspex block surrounding a tubular extension at the base of the heart chamber. A weighted ferrite core (3.2g) was attached to the apex of the heart, the vertical movement of this core providing a measure of myocardial contraction. The response of this device was linear over the range used and a pen deflection of 6.3mm was equivalent to 1mm movement of the core. Isotonic contraction has been used by various workers as a measure of ventricular function (Lu and Melville, 1951; Zachariah, 1961; Nayler and Emery, 1962). The heart chamber and transducer are illustrated diagrammatically in figure 6.

Perfusion fluid entered the coronary circulation from the aorta and passed via the coronary veins to the right side of the heart from which it emptied. Coronary flow was calculated by conversion of the number of drops per min., measured by a drop counter placed at the outlet from the heart chamber, to ml per minute.

Outputs from the differential transformer and the drop counter were recorded on a Beckman type RP dynograph recorder.

All preparations were perfused for a twenty minute equilibration period and experiments were then conducted over the following 90 minutes. After the initial equilibration period the parameters measured remained constant for approximately two hours. The recording showed no appreciable base line shift representing overall contraction or relaxation of the heart over this period.

The normal perfusate was bubbled with 95% O_2/5% CO_2 and was filtered through a sintered glass filter in the perfusion reservoir (Bleehen and Fisher, 1954). In some experiments 1% dextran (Macrodex, Pharmacia) was added to the bathing solution as a possible method of reducing oedema in the heart. However, it appeared to have little or no effect and so was omitted in later experiments. Ascorbic acid (0.1mM) was

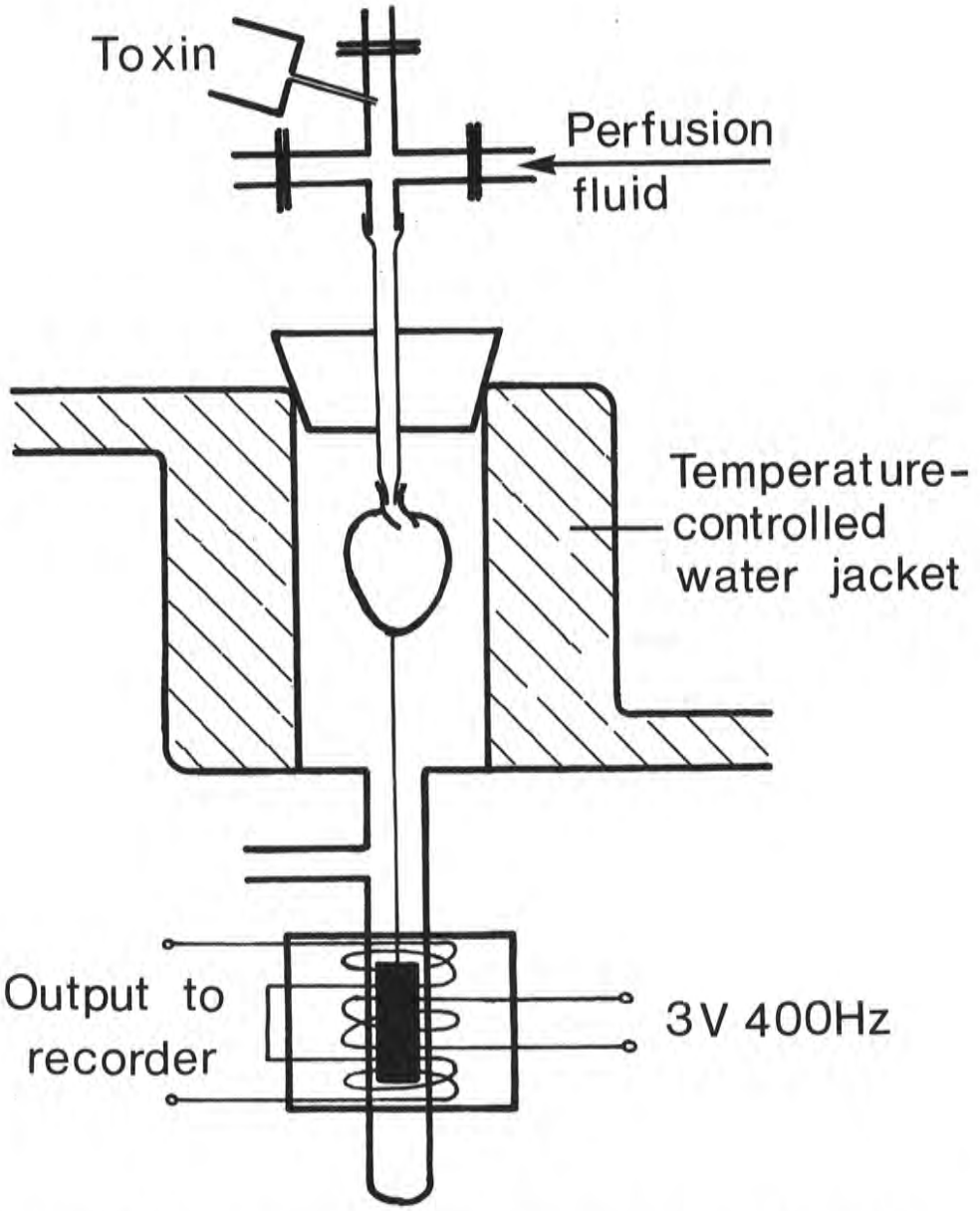


Figure 6: Diagram of heart chamber and differential transformer transducer. The three-way connector above the chamber allows entry of perfusion fluid from either of two sources; the third arm is for removal of gas bubbles. The differential transformer could be moved in a vertical plane to enable alignment with the ferrite core.

added to all solutions for experiments involving catecholamines (Satchell, Freeman and Edwards, 1968). This level of ascorbic acid had no observable effect on the parameters measured. The temperature of the perfusate was 37.0 ± 0.3°C.

Crude Chironex toxin was divided into 0.5 ml ampoules and stored at -15°C so that small amounts could be thawed and used at any one time. For the dose-response data, separate samples were thawed and diluted for each level as required. The potency of these extracts was checked several times throughout the series to ensure that the dose of toxin remained constant. Sephadex G-200 fractions were checked for lethality immediately before use.

The toxin sample was added to the perfusion fluid at a point just proximal to the heart. As there was no recirculation there was only one contact between the heart and the toxin; recirculation, however, would have had little effect as diluted Chironex toxins are extremely labile at 37°C.

As the response to Chironex toxin, at the level used, was transient and therefore did not reach a steady state, modification of the toxin response by drugs could only be investigated by measurement of the changes in cardiac parameters produced by the toxin in the presence of a steady response to the particular drug being used. The dose levels of catecholamines and other drugs were therefore chosen in preliminary experiments as representing the minimum dose consistent with a steady response of reasonable magnitude. The doses of alpha and beta blocking drugs were minimum blocking doses.

In all experiments the changes in cardiac parameters are expressed as percentages. These were calculated using the control level over 2 - 3 minutes prior to addition of either the toxin or the vasodilator as 100%. The percentage changes in flow, rate and amplitude due to Chironex toxin in

the presence of a vasodilating agent are calculated using the steady state levels obtained in the presence of the vasodilator as 100%.

Perfused Rabbit Ear. The methods of de la Lande, Paton and Waud (1968) and de la Lande, Cannell and Waterson (1966) were used. The rabbit was anaesthetized with a Fluothane: N_2O: O_2 mixture and the central ear artery cannulated at the base of the ear. 10 - 20 ml heparinized saline was perfused by syringe and the ear removed and placed inside a glass coil maintained at the perfusion temperature of 37°C. Perfusion was commenced immediately and was kept at a constant rate with a peristaltic pump.

Preparations of the central ear vein or artery were isolated similarly. 3 - 4cm segments of vein or artery were cannulated at both ends and perfused in the direction of blood flow. This method isolated the fluid passing through the vessel lumen from the bulk of the bath fluid. The absence of leaks between the lumen and the exterior was tested by perfusion of a dye solution through the vessel at the end of the experiment.

Perfusion pressure was measured with a Statham p23Db transducer coupled to a Beckman RP dynograph recorder. Flow rate in the whole ear and ear artery was 7.5 ml/minute and the average perfusion pressures were 45mm Hg (ear) and 42.5mm Hg (artery). Flow rate in the vein was 2 ml/minute and the average perfusion pressure was 10mm Hg.

Rabbit aorta. The method described by Furchgott and Bhadrakom (1953) was used. The thoracic aorta was removed under Fluothane: N_2O: O_2 anaesthesia and placed in a dish of bathing solution. After trimming excess fat and connective tissue the aorta was cut along a close spiral to yield a strip approximately 2 mm wide and several centimetres long. A 2 - 4cm segment was mounted in a 20 ml bath and allowed to equilibrate for approximately 1.5 hours. Isotonic contractions were measured in the presence of a constant load (3.2g)

with a differential transformer modified from that used in the isolated heart experiments.

MICROELECTRODE RECORDING

Several experiments required the measurement of the membrane potential of denervated rat diaphragm muscle before and after addition of drugs. This was achieved by intracellular recording with a glass micropipette electrode.

Microelectrodes were made from 2.5mm glass tubing (Micropet, Clay Adams) and filled with 3M KCl under reduced pressure. The average value for tip resistance was 9 megohms (range 6 - 20 megohms) and tip potentials were less than 10mV (Thesleff, 1963). The microelectrode was attached by a Perspex holder to a Singer micromanipulator and was connected through a Ag/AgCl electrode to a preamplifier (200 megohms input resistance). Preamplifier output was fed into a Tektronix 564 oscilloscope and a Beckman RP dynograph recorder.

A calibrated millivolt source between the indifferent bath electrode and earth permitted rapid measurement of the membrane potential by acting as an opposing potentiometer. The potential difference, read when the current was zero, was a direct measure of membrane potential. Readings were made to the nearest millivolt.

The diaphragm muscle was pinned to a silicone rubber block (Rhodorsil) which covered the base of the 5 ml organ bath. Bath temperature, unless otherwise stated, was maintained at 29 - 30^{o}C. The normal flow rate through the system was 2 ml/minute, but this could be increased to 4 ml/minute for washout of a drug. The bath volume was

controlled by a suction overflow tube. To obtain an estimate of thetime required for clearance of a drug, a dye dilution method was used. Serial samples (0.2 ml) were taken from the bath during an emptying cycle and diluted to 2 ml for optical density measurement with a Unicam SP-600 spectrophotometer against a nutrient solution blank. Conversion of optical density values to dye concentrations on a standard curve showed that the decrease in dye concentration was approximately exponential. The time to half clearance (t_{50}) was 28 - 30 seconds; t_{99} was approximately 3.5 minutes.

A PHARMACOLOGICAL STUDY OF THE TOXINS ISOLATED FROM THE CNIDARIAN, CHIRONEX FLECKERI

The Cubomedusae, or box jellyfish, have been implicated in many injuries to man in Northern Australian waters. Two species, Chironex fleckeri and Chiropsalmus quadrigatus, have been responsible for approximately sixty deaths and many more non-lethal stingings. Fatalities have usually been reported between November and February when the jellyfish inhabit the shallow coastal waters. In his account of these two species, Barnes (1966) suggests that Chironex is more dangerous than Ch. quadrigatus because of its larger size and greater number of nematocysts. Both species, however, are capable of inflicting dangerous and extremely painful stings.

With the increasing development of Northern Australia to tourism and industry, it is likely that the incidence of stingings will rise, thus increasing the already existing health hazard.

Until recently, no detailed studies of Chironex fleckeri toxin had been made. However, over the past three years, reports from a number of workers have described several aspects of the toxin and its actions. The aim of the experiments which are described in the following section has been to investigate the pharmacological properties of the Chironex toxin fractions which have been prepared. In order to examine fully the effects of the toxin in both intact animals and isolated organs, a considerable range

of preparations and techniques has been employed. The results have been arranged so that experiments with intact animals, including mouse toxicity tests and anaesthetized animal experiments, are described first. Several aspects of the responses observed are then studied further in experiments with isolated organ preparations.

The responses of experimental animals to injections of toxin extracts closely resemble those described in clinical reports of Chironex stingings in humans. Death was due to respiratory arrest of central origin, associated with marked signs of cardiovascular failure.

Although not included in this report, some studies of the pharmacological properties of Chiropsalmus quadrigatus toxin have also been made (Freeman and Turner, in preparation). These have not revealed any significant differences from Chironex toxin.

RESULTS

Experiments with several Chironex toxin preparations are described in this section. The fractions obtained by Sephadex G-200 chromatography are referred to most conveniently as the 'cardiotoxin' and the 'haemolysin'. The cardiotoxin (M.W. 150,000) is lethal but not haemolytic; the haemolysin (M.W. 70,000) possesses haemolytic and lethal activity and causes skin necrosis upon intradermal injection. The 'crude'toxin includes supernatant fractions of centrifuged tentacle extracts as well as samples prepared by Sephadex G-75 chromatography of these fractions. G-75 chromatography did not alter the properties of the centrifuged extract.

LETHAL EFFECTS OF CHIRONEX TOXIN

Mice exhibited similar symptoms after intravenous injection of lethal doses of crude toxin, 'milked' toxin or either of the Sephadex G-200 fractions. The animals became lethargic and ataxic, respirations appeared forced and irregular, and before death the animals convulsed briefly, but often quite violently. At autopsy the heart was usually beating feebly and irregularly and there was marked venous engorgement. The lungs were reddish-orange in colour.

Mice rarely died earlier than sixty seconds after a lethal injection and deaths later than thirty minutes were unusual. The time to death after injection of haemolysin was longer than that observed after any of the other toxin

extracts. Dose mortality curves for all extracts were extremely steep.

The effects of lethal doses of toxin on anaesthetized rats and rabbits followed a similar pattern to those seen in mice, except that the anaesthetized animals did not convulse. Autopsy findings were also similar.

LETHAL TOXIN LEVELS

Crude toxin. The quantity of crude toxin required to kill a rabbit by intravenous injection was extremely variable, ranging from 10 - 160 M.U. The shortest time to death was 90 seconds, the longest was 6 minutes. 2 - 4 M.U. was the rat lethal dose; the time to death varied from 2 - 10 minutes.

Haemolysin. Rabbits died 5 - 12 minutes after doses ranging from 10 - 15 M.U. injected into the marginal ear vein (4 rabbits); rats died 5 - 45 minutes after injection of 2 - 9 M.U. into the lateral tail vein (9 rats). Doses were injected over a standard ten second period. Rabbits recovering from near-lethal doses had a depressed arterial pressure for some 20 minutes after the injection, but late deaths after 30 - 40 minutes were not seen.

Cardiotoxin. Rabbits died 3 - 5 minutes after doses of cardiotoxin ranging from 8 - 12 M.U. (4 rabbits); rats died 5 - 40 minutes after injection of 0.3 - 2.5 M.U. (9 rats). All injections were administered over a standard ten second period. The results were qualitatively different in the two species in that symptoms persisted in the rat

after near-lethal doses for 30 - 40 minutes, whereas, under the same circumstances, the rabbit showed complete recovery after 5 - 8 minutes.

EFFECTS OF CHIRONEX TOXIN ON ANAESTHETIZED ANIMALS

CARDIOVASCULAR SYSTEM

A similar pattern of effects was observed after intravenous injection of any of the toxin preparations into rats or rabbits. Control values for all measurements are shown in Table 4.

Table 4. Control levels of physiological parameters in rabbits and rats.

	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	Right atrial pressure (cmH_2O)	Heart rate (min^{-1})	Respiratory rate (min^{-1})
Rat	161±4 (33)	127±3 (33)	0.2±0.4 (33)	372±10 (33)	74±3 (33)
Rabbit	125±4 (10)	91±4 (10)	-0.2±0.7 (10)	300±8 (10)	39±3 (10)

Figures shown are ± S.E.M. The number of observations is shown in parentheses.

These have been calculated from the results of experiments with the G-200 fractions only, but readings from all experiments fall within these ranges.

Effective doses of Chironex toxin invariably caused a biphasic change in arterial pressure in both rabbits and rats. Within 30 seconds after injection of crude toxin,

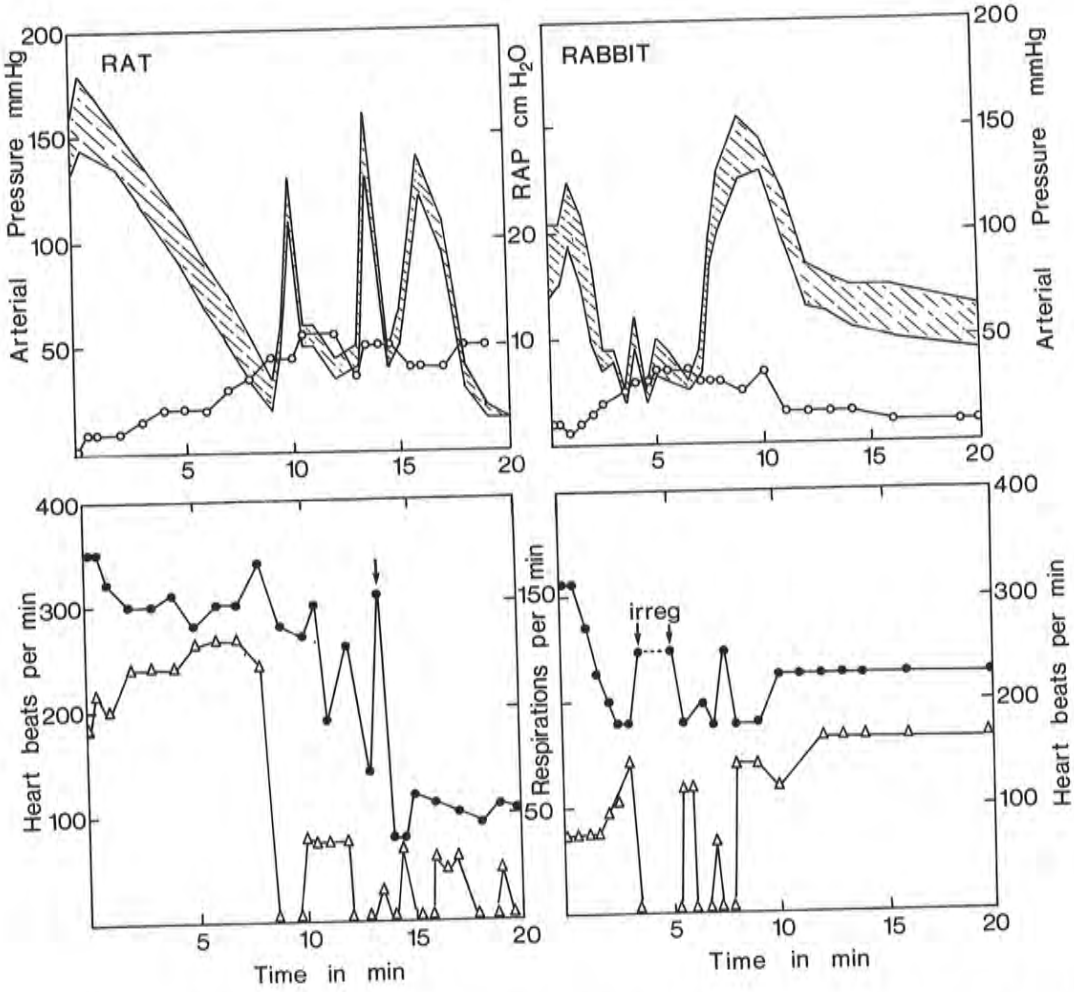


Figure 7: The effect of Chironex haemolysin on arterial pressure (hatched area), right atrial pressure (O——O), heart rate (●——●) and respiratory rate (Δ——Δ) in rat and rabbit. The rat received 4 M.U. at zero time; recovery was complete after 30 minutes. The rat had an episode of ventricular tachycardia at 14 minutes (arrow); the heart rate in the rabbit showed marked irregularities from 3 - 5 minutes.

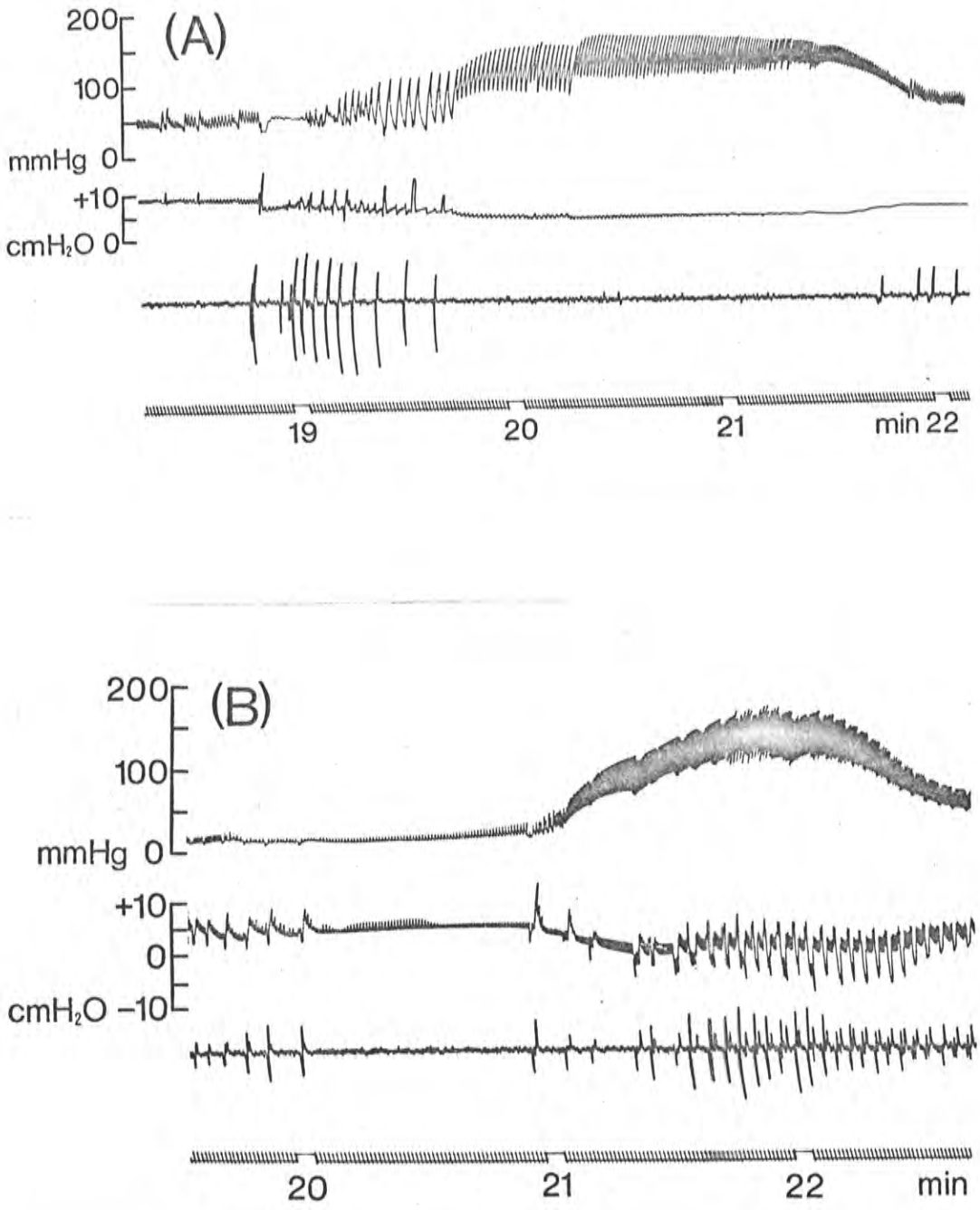


Figure 8: The effect of haemolysin (A) and cardiotoxin (B) given at zero time on arterial pressure (upper), right atrial pressure (middle) and respirations (lower). Haemolysin (8 M.U.): the rat was moribund at 19 minutes; after a period of gasping respirations, all parameters improved, although the animal was apnoeic for 2 minutes. Cardiotoxin (0.6 M.U.): the animal had been bilaterally vagotomized. Here the rise in arterial pressure was coincident with the return of respiratory activity.

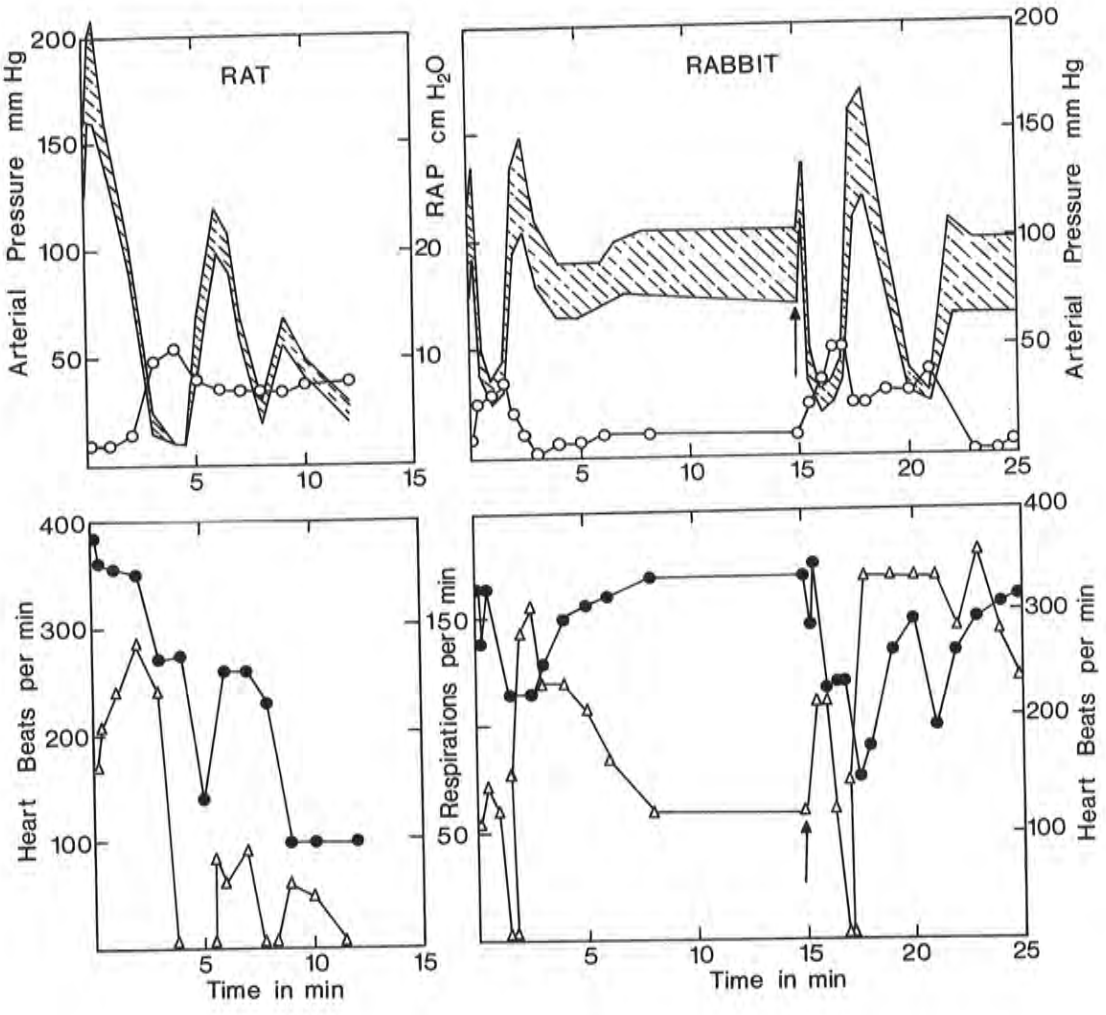


Figure 9: The effect of Chironex cardiotoxin on arterial pressure (hatched area), right atrial pressure (O——O), heart rate (●——●) and respiratory rate (Δ——Δ) in rat and rabbit. The rat received 1 M.U. at zero time, the rabbit received 3 M.U. at zero time and 6 M.U. at 15 minutes. The rabbit recovered.

'milked' toxin or cardiotoxin the arterial pressure rose by 25 - 60 mmHg. A similar rise of 20 - 30 mmHg, over a 60 second period, was observed after injection of haemolysin.

The rise in blood pressure was followed by a rapid fall over the next few minutes. This fall, which was usually greater in the rat than the rabbit, was associated with the development of bradycardia, cardiac irregularities and a rise in right atrial pressure. During this period there was always some acceleration of the respiratory rate followed by a rapid diminution in respiratory amplitude and apnoea of variable duration. In most instances, a dramatic rise in arterial pressure, often to above the control level, was associated with the first period of apnoea. Thereafter, the arterial pressure decreased once more. In some animals the second fall in arterial pressure preceded the terminal apnoea; in others a series of violent pressure oscillations occurred before death. Typical experiments are illustrated in figures 7 (haemolysin) and 9 (cardiotoxin); portions of recorder trace illustrating the pressure oscillations are shown in figure 8.

The time scale of events varied from animal to animal, but the pattern was always similar unless a very large dose of toxin was given, when death occurred during the first fall in arterial pressure.

The electrocardiographic changes observed after the injection of crude toxin, 'milked' toxin, or either of the Sephadex G-200 fractions followed similar patterns in both rats and rabbits. Bradycardia was seen within one minute

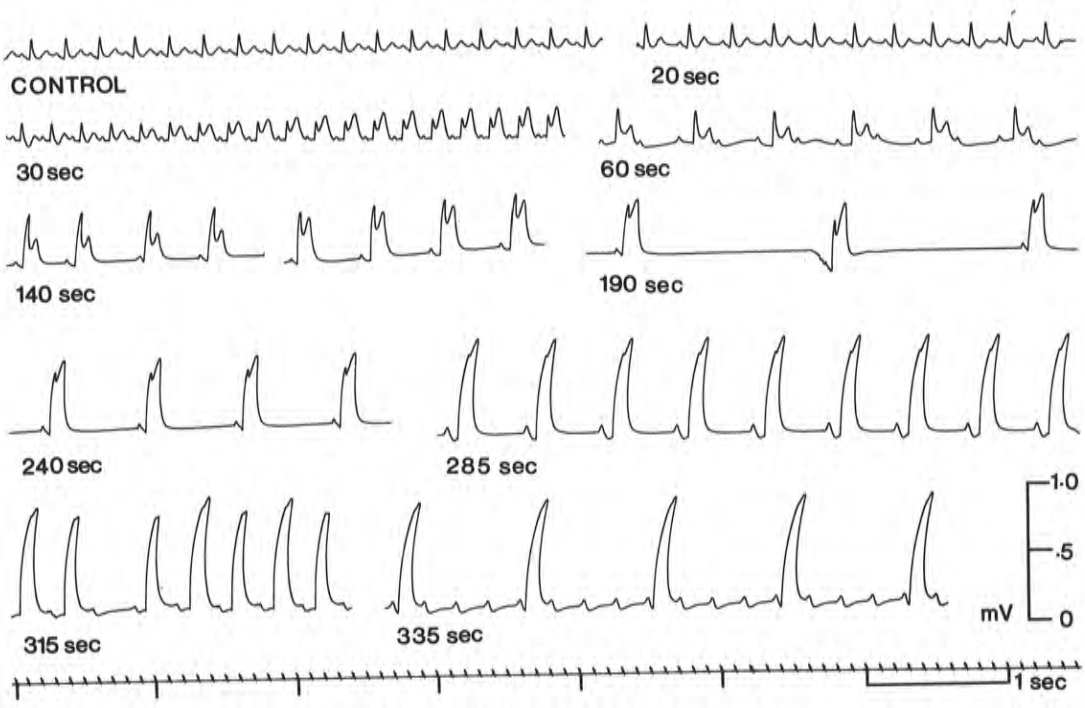


Figure 10: Electrocardiographic changes following injection of crude toxin. The first panel shows a segment of ECG before injection, heart rate 260 per minute. There is an increased T-P interval and bradycardia 20 seconds post-injection. T-wave enlargement is seen after 30 seconds. At 60 seconds there is 2 : 1 atrioventricular block. Between 140 and 190 seconds respirations became irregular and gasping. At 315 seconds the heart showed irregular a-v block; the animal ceased to breathe at 320 seconds.

of toxin injection. Some T wave enlargement was noted; this was especially marked after injections of the crude toxin. S-T segment depression was commonly observed 2 - 3 minutes after administration of the Sephadex G-200 fractions. Thereafter, traces showed T wave inversion, conduction delay, ventricular tachycardia and intermittent atrioventricular block. Irregular ECG patterns were sometimes interspersed with more or less regular periods of atrioventricular block; heart rate was occasionally observed to decrease and increase several times before death.

Typical ECG tracings are shown in figure 10. These were obtained from an anaesthetized rabbit before and after injection of a lethal dose of crude toxin.

RESPIRATORY SYSTEM

None of the toxins altered respiratory rate or amplitude until after the first hypertensive period when arterial pressure was falling. During this period there was an increase in respiratory rate followed by a rapid decrease in amplitude, and apnoea of variable duration; the extreme range of apnoea noted in both species was 20 - 120 seconds. As the blood pressure began to rise to normal levels, the respiratory rate returned to control values or above. Similar periods of respiratory depression or apnoea accompanied each pressure oscillation. One rabbit which had received a dose of haemolysin recovered after three long (100 seconds) periods of apnoea interspersed with periods of hyperpnoea. In other animals apnoea was followed by periods of slow, gasping respirations

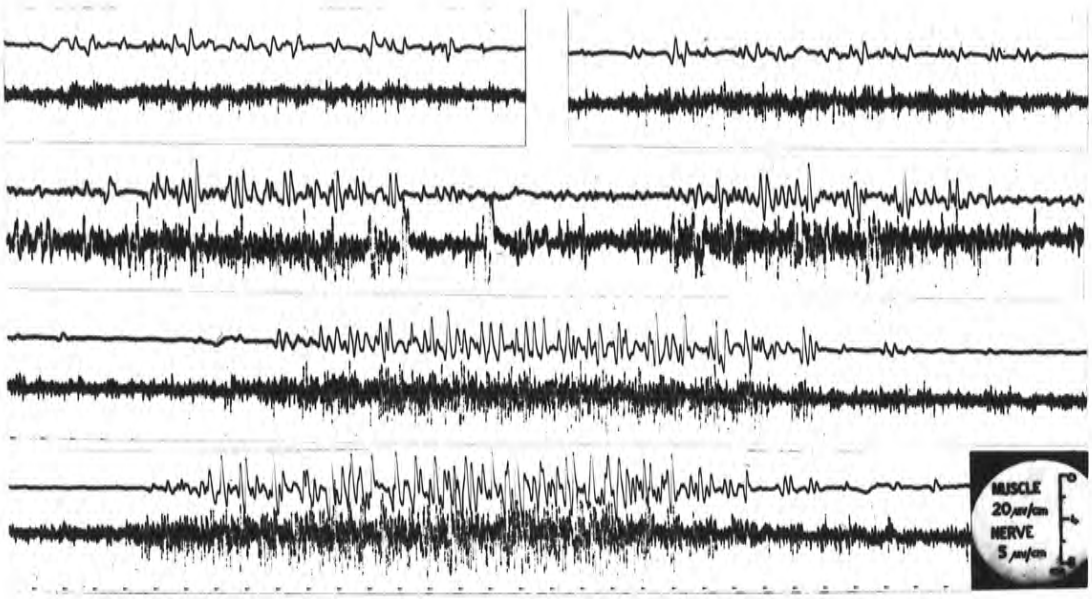


Figure 11: Phrenic nerve and diaphragmatic activity following injection of crude Chironex toxin. Upper trace shows record from myograph needle in the diaphragm, lower trace shows phrenic impulses. Panel 1, control respiration; Panel 2, increased phrenic activity after a non-lethal toxin injection; Panel 3, irregular gasping respirations after a lethal dose; Panels 4 and 5, exaggerated activity associated with slow, gasping respirations. Panel 5 shows the terminal gasp, which was followed by electrical silence. Time marker: 0.02 seconds.

then death.

In experiments with the crude toxin, short periods of apnoea were sometimes observed as the blood pressure increased following a hypotensive episode. Such periods of apnoea were never observed after injection of haemolysin or cardiotoxin. A small number of rats showed pulmonary oedema. This was only observed in the rat and could occur after administration of any of the toxin extracts.

Respiratory changes after the crude toxin were further investigated by monitoring simultaneously the electrical activity in the phrenic nerve and the diaphragm muscle. Figure 11 shows the results of a typical experiment. Panel 1 illustrates the electrical activity associated with one normal respiration. Inspiration lasted for approximately 300 msec; the respiratory pause was 550 msec. Panel 2 shows the increased respiratory rate after a non-lethal dose of toxin. The duration of inspiration was unchanged; the increased rate was due to a decrease in the respiratory pause. Subsequent panels illustrate the effects of a lethal dose of toxin. Gasping, due to a lengthening of inspiration is shown in panel 4. At this time respiration appeared to be entirely diaphragmatic and, as illustrated in the myograph, there was an augmented force of contraction. Panel 5 illustrates the terminal gasp before apnoea and death. These records show that neuromuscular transmission remained effective until death. This was confirmed by placing stimulating electrodes on the nerve post mortem, when it was possible to elicit a vigorous diaphragmatic twitch.

It is clear that the respiratory centre remained capable of initiating respiratory signals until the terminal respiration.

EFFECTS OF CHIRONEX TOXIN ON VASOMOTOR REFLEX PATHWAYS

The question arises as to whether the arterial pressure oscillations which are a constant feature of death are due simply to fluctuations in cardiac function against a constant background of vasoconstriction or to some more complex mechanism. The oscillations suggest a disordered feedback system, and raise the possibility that the toxin has brought about a disturbance of vasomotor reflexes. Consequently, experiments were designed to test the possibility that Chironex toxin interferes with some part of the vasomotor reflex pathway. In most of these experiments the cardiotoxic and haemolytic fractions prepared by Sephadex G-200 chromatography have been used. Where crude toxin extracts have been administered, these results are also described.

Effects on carotid occlusion reflexes. Carotid occlusion reflexes were elicited by clamping one or both of the carotid arteries for 30 seconds. Both the carotid arteries of the rabbit could be clamped as the femoral artery had been cannulated. In the rat, however, only the left artery was clamped as the right carotid had been cannulated for blood pressure recording.

The effects of haemolysin and cardiotoxin on this reflex were determined after low doses, so that periods of relatively stable hypotension lasting 4 - 5 minutes

were available after the initial hypertensive response. Attempts were made to elicit the reflex when the arterial pressure in the rat varied from 47 - 70 mmHg, respirations varied from 25 - 65 per minute and the heart rate varied from 260 - 380 per minute. It was not possible after either toxin to elicit a pressor response to carotid occlusion, although l-noradrenaline bitartrate (1 - 2µg/kg) given immediately after such attempts caused an increase in the arterial pressure of approximately 70 mmHg. Noradrenaline given close to death was ineffective, however, possibly because of the generalized circulatory failure.

In the rabbit the carotid occlusion reflex was negative for 5 - 10 minutes after a non-lethal dose of either toxin. Due to the short duration of symptoms after the cardiotoxin, the arterial pressure had returned to a near normal level before the carotid occlusion reflex was tested.

Because of the sensitivity of carotid occlusion reflexes to hypoxaemia, a series of experiments was conducted in which rabbits were ventilated with a Palmer respiratory pump throughout the experimental period. Spontaneous respirations were suppressed with d-tubocurarine (0.2mg/kg) given as required.

Non-lethal doses of cardiotoxin produced changes which were similar to those observed in the non-ventilated rabbit. The second rise in arterial pressure was observed only after a prolonged period of hypotension (nearly 2 minutes), which would presumably produce a sufficient degree of hypoxia to excite chemoreceptors despite the

maintenance of pulmonary ventilation.

Carotid occlusion reflexes could not be elicited for periods of 4 - 8 minutes after cardiotoxin injection although systolic pressure was 90 - 120 mmHg. Suppression of the reflex was not dependent on a prior episode of hypotension which may have depressed the vasomotor centre.

Effect of vagotomy. Bilateral cervical vagotomy reduced the resting respiratory rate in both rats and rabbits. Injections of crude toxin, cardiotoxin or haemolysin after vagotomy produced similar patterns of results to those previously described except that at no time was there a significant increase in respiratory rate. Arterial pressure oscillations developed in the usual way, although the clear-cut relationships between apnoea and the pressor episodes was lost. As may be seen in figure 8B apnoea sometimes preceded the arterial pressure increase, which was coincident with the return of respiratory activity.

Carotid sinus denervation in the rat. The superior laryngeal, recurrent laryngeal, aortic depressor and vagus nerves and the sympathetic trunk were sectioned, together with the nerves arising in the carotid bifurcation in the contralateral carotid to the arterial cannulation. The arterial pressure in such rats always exceeded 200 mmHg but was rather unstable, and even the stimulation caused by insertion of the right atrial cannula could result in a prolonged period of hypotension. One rat died in this way, although at autopsy there was no evidence to suggest that the cannula had perforated either the vein or the atrium. Another rat suffered a precipitous fall in arterial pressure following insertion of the atrial cannula;

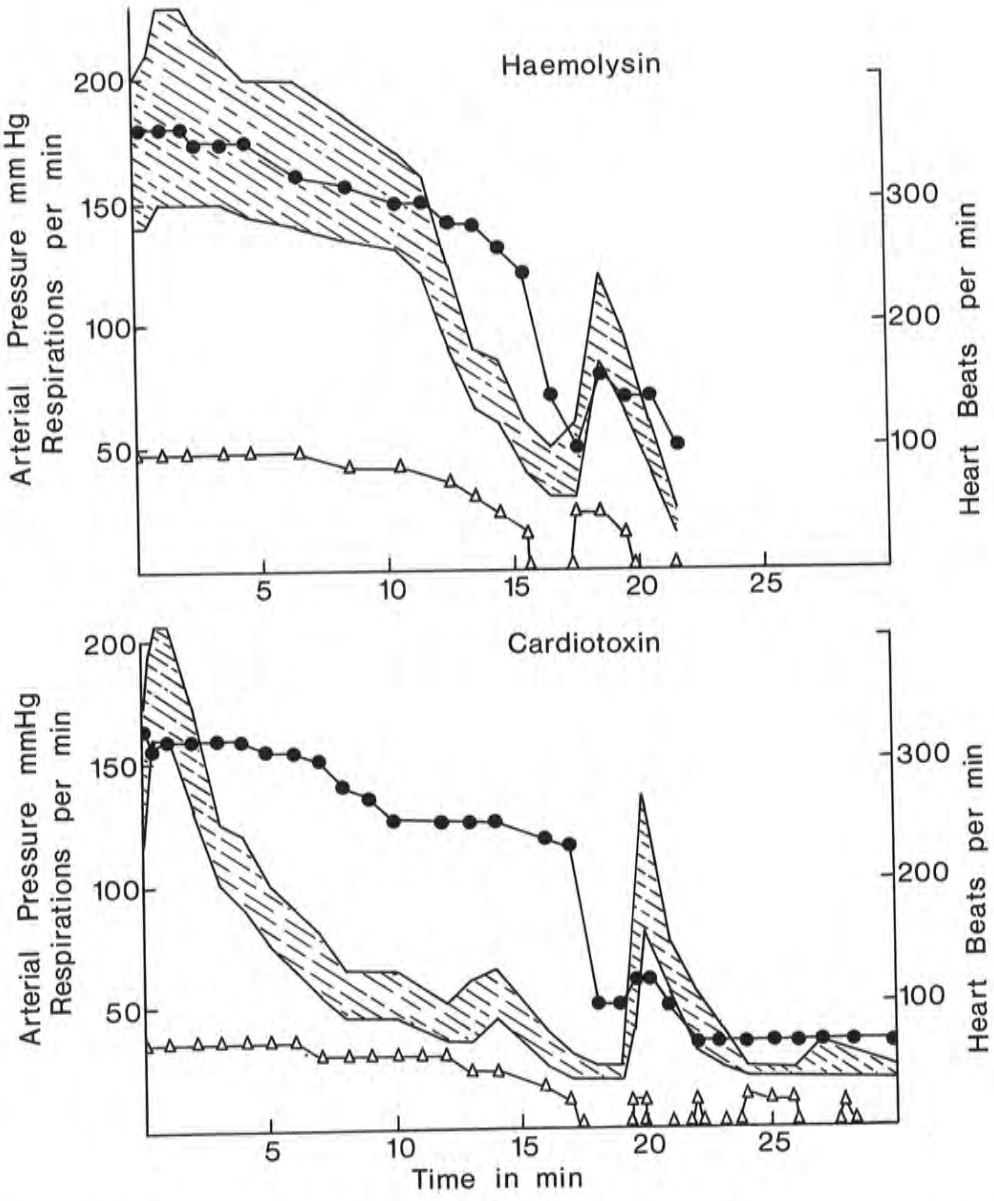


Figure 12: Effect of Chironex haemolysin and cardiotoxin after radical section of the sinoaortic afferents in the rat. Upper diagram: Haemolysin (5 M.U.). Lower diagram: Cardiotoxin (2.5 M.U.). This animal died at 30 minutes. Hatched area, systolic and diastolic pressure; ●——● Heart rate; Δ——Δ respiration rate.

over a 40 second period, arterial pressure fell from 195mm Hg to 30mm Hg. At this point the animal stopped breathing for 90 seconds. Coincident with the return of respiratory activity the pressure rose again, and a series of slow pressure oscillations preceded death 40 minutes later. Each rise in arterial pressure was triggered off by a period of apnoea. It would seem that, without the buffering action of peripheral baro- and chemo-receptors pressure oscillations, which resemble those induced by the toxins, can occur. Because of the hazards of atrial cannulation this was omitted from this series of experiments.

Although the rats were hypertensive after baroreceptor denervation the injection of either cardiotoxin or haemolysin produced a further increase in arterial pressure by 10 - 30mm Hg. This was followed by a slower than usual fall in pressure and a period of apnoea, which was succeeded by one or two pressure oscillations. As may be seen from figure 12, denervation reduced the respiratory rate from the control value of 74 per minute (Table 4) to 54 $\pm$ 5 (S.E.M., 8 rats). The heart rate was not significantly altered. Cardiac and respiratory difficulties developed in the usual way after injection of either toxin, except for the absence of respiratory stimulation prior to apnoea. However, unlike rats with baroreceptor innervation intact, the pressor response to apnoea did not occur until respirations had resumed. This was a constant finding with both toxins.

Effect of hexamethonium. If the arterial pressure oscillations are due to factors additional to fluctuation in cardiac

function, then interference with any part of the vasomotor reflex pathway should reduce or abolish such oscillations. The ganglion-blocking drug, hexamethonium, was injected into rabbits and rats in divided doses of 2.5mg/kg until the arterial pressure had fallen to a steady level, and it was no longer possible to elicit a carotid occlusion reflex. Usually a total dose of 10mg/kg was given over a 20 minute period. The arterial pressure in the rat was reduced to 91 ± 6mm Hg (S.E.M., 9 rats).

Injection of cardiotoxin or haemolysin after ganglionic blockade brought about the usual pressor response, which averaged 60mm Hg above the control level. Thereafter, the arterial pressure fell; pressure oscillations were either entirely absent or were slow, small in magnitude (20 - 30mm Hg), and unrelated to periods of apnoea. Respiratory and electrocardiographic changes followed the usual pattern. Closely similar results were obtained with rabbits which had received either of the Sephadex G-200 toxins or crude toxin after ganglionic blockade with hexamethonium.

These results suggest that the initial pressor response is due to a direct vasoconstriction, although such a response could also be mediated by catecholamine release, in a manner similar to that of the indirectly acting sympathomimetics, tyramine and B-phenylethylamine.

Effect of phenoxybenzamine. The possibility of catecholamine release was tested in rats and rabbits pretreated with phenoxybenzamine hydrochloride (2 - 4mg/kg). The animals received the alpha adrenergic blocking agent intravenously two hours before the administration of either cardiotoxin

or haemolysin. These doses completely inhibited the carotid occlusion reflex and the response to noradrenaline. The arterial pressure was reduced to 117 ± 7mm Hg (S.E.M., 6 rats) and 70 - 80mm Hg (4 rabbits). Right atrial pressure, heart rate and respiratory rate were not significantly altered in either species.

After phenoxybenzamine the initial pressor response to either toxin was increased in both species; it was also sustained for longer after both toxins in the rat. At no time were pressure oscillations seen. In the rabbit the initial pressor response was followed by a rapid fall in arterial pressure after both toxins. This was followed by a period of apnoea of 20 - 30 seconds duration. As respirations resumed, the arterial pressure rose again to slightly higher than the control level, and then slowly returned to normal. The hypotensive episode was always associated with a sharp but transitory rise in right atrial pressure, suggesting that the hypotension was due to a temporary fall in cardiac output. After lethal doses of either toxin the rabbit died during this first hypotensive period, without showing any further pressure oscillations.

The lethal dose of haemolysin in the rabbit was raised from 10 - 15 M.U. to 50 - 60 M.U. after pretreatment with phenoxybenzamine. There was a similar, but less clear cut, tendency in the rat.

Effect of bretylium. Intravenous injection of bretylium tosylate (10mg/kg) into rats some 20 - 30 minutes before toxin injection did not affect the initial pressor response to either of the Sephadex G-200 toxins. Hypotension and an increase in right atrial pressure followed this response.

The rate of decline of arterial pressure was dose dependent, and there were no pressure oscillations associated with the apnoeic periods.

Effect of propranolol. Intravenous injection of propranolol at a level of 0.1mg/kg did not appear to affect the response of the rat to Chironex haemolysin or cardiotoxin. However, 0.4mg/kg propranolol markedly damped the arterial pressure oscillations. This may reflect the local anaesthetic action of this dose level of the beta adrenergic blocking agent (Shanks, 1967).

PLASMA ELECTROLYTE LEVELS

The ECG abnormalities which typically included T wave elevation followed by inversion, bradycardia and conduction delay could be explained by hypoxia, a release of K^+, or both.

Consequently, blood samples were taken from rabbits and rats which had received crude Chironex toxin before respiratory arrest. There was a variable amount of haemolysis of the heparinized samples, which could be correlated with the plasma K^+ level. Terminal K^+ levels ranged from 5.8 mmoles/l to 19.2 mmoles/l. No significant change in plasma Na^+ was noted.

HISTAMINE RELEASE FROM THE SKIN

The cutaneous wealing observed after human envenomation may be due to release of vasoactive substances by the toxin. The possibility of histamine release from the skin was investigated by injection of Chironex toxin into a rat skin pouch. Control and test washings were

obtained from the skin pouch as described in the Methods. All wash samples produced slight responses in the guinea pig ileum assay preparation, which could be blocked by prior addition of diphenhydramine. This suggested that histamine was present (1 - 2ng per ml), but in no experiment was the histamine content increased by treatment with the toxin.

EFFECTS OF CHIRONEX TOXIN ON ISOLATED ORGAN PREPARATIONS

Several aspects of the response of anaesthetized animals to the toxin were examined further by the use of isolated organ preparations. Perfused heart and blood vessel preparations provided further information on the effects of the toxin on the cardiovascular system; the effect of the toxin on smooth muscle was observed in isolated preparations of guinea pig ileum and trachea.

Both crude extracts and chromatographed extracts were used in this series of experiments to indicate whether part of the response was due to the presence of pharmacologically active substances of low molecular weight. As suggested previously, the wealing which follows Chironex stingings in humans could possibly be related to the injection or release of histamine in the skin; the pain associated with stingings could be due to the presence of 5-hydroxytryptamine or acetylcholine.

RAT PHRENIC NERVE-DIAPHRAGM PREPARATION

Because of the occurrence of respiratory failure, a number of experiments were carried out to test the effects

of crude and 'milked' toxin on the diaphragm preparation. Doses of toxin equivalent to 10 M.U./ml increased the resting tension of the preparation for approximately ten minutes. Thereafter, there was a slight decline in twitch tension following direct or indirect stimulation. This effect could not be reversed by washing, but did not alter the response of the preparation to succinylcholine or d-tubocurarine.

DENERVATED RAT DIAPHRAGM

Chironex toxin did not affect the response of the denervated diaphragm to acetylcholine, nor was the rate of spontaneous fibrillation of the diaphragm altered. The toxin appears to lack any specific anticholinergic effect.

GUINEA PIG ILEUM

Both crude and 'milked' toxins consistently produced a slow contraction of the guinea pig ileum. A second dose of toxin after washing the preparation for 15 minutes produced a much smaller contraction and it was frequently impossible to obtain a third response. Exposure of the preparation to the toxin markedly reduced its sensitivity to 5-hydroxytryptamine or histamine. Large doses of toxin (2 - 3 M.U./ml) were necessary to produce a contraction.

GUINEA PIG TRACHEA

2 - 3 M.U./ml of crude toxin produced slow constrictions of the isolated trachea. These were small in magnitude and could be obtained only once or twice in any preparation. They appeared to be blocked by diphenhydramine (2×10^{-8} g/ml),

but the difficulty of obtaining repeated responses to the toxin made assay experiments impossible.

HISTAMINE RELEASE FROM MAST CELLS

Doses of crude toxin ranging from 1 - 5 M.U./ml brought about some histamine release from mast cell preparations. However, microscopic examination of the mast cells showed that histamine release could be correlated with cell damage, suggesting that the toxin may produce a non-specific disruption of cell membranes. This could also account for the action of the toxin on the rat diaphragm and the smooth muscle preparations.

PERFUSED GUINEA PIG HEART

Effects of Chironex toxin on the untreated heart.

The control values for coronary flow, heart rate and amplitude were consistent throughout the series of experiments. Coronary flow had a mean rate of 10.5 $\pm$ 0.5 ml per minute ($\pm$S.E. of 56 observations) and mean heart rate was 214 $\pm$ 4 beats per minute ($\pm$S.E. of 56 observations). These values are consistent with those of other workers (Westfall, 1968). The height of the recorded amplitude trace is proportional to the peak amplitude of the isotonic contraction. This parameter is convenient to measure and appears to be linearly related to other indices of ventricular function (Opie, 1965). A mean value of 21.0 $\pm$ 0.5mm deflection ($\pm$S.E. of 56 observations) was obtained.

The injection of a standard dose (0.03 M.U.) of crude toxin or cardiotoxin into the coronary perfusion circuit brought about reversible changes in the three parameters

being measured. There was an immediate decrease in coronary flow rate to approximately 50 per cent of control levels. This persisted over the first minute and was followed by a slower return to the control level over the next 4 - 7 minutes. The decrease in coronary flow was accompanied by a slight bradycardia; the heart rate decreased over the first 2 - 3 minutes and then returned to control levels within 10 minutes of toxin administration. A marked reduction in amplitude was also observed; this followed a similar time course to the rate change.

In contrast with the other extracts, low doses of Chironex haemolysin showed little effect on the isolated heart preparation. None of the parameters was reduced by more than 5 per cent after injection of the standard 0.03 M.U. dose.

None of the toxin fractions produced a significant overall contraction or relaxation of the heart. The effects of the three fractions are illustrated in figures 13 and 15. Injections of toxin were separated by periods of 10 - 15 minutes throughout the course of all experiments. Coronary flow and heart rate changes were reproducible both within experiments and between experiments, but amplitude changes were subject to some variation. In figure 13, the amplitude changes in response to the first and second doses of crude toxin are identical but the third dose has had little effect. In most experiments, however, the degree of variation was less than this.

Dose-response relationships.

The dependence of changes in coronary flow, heart

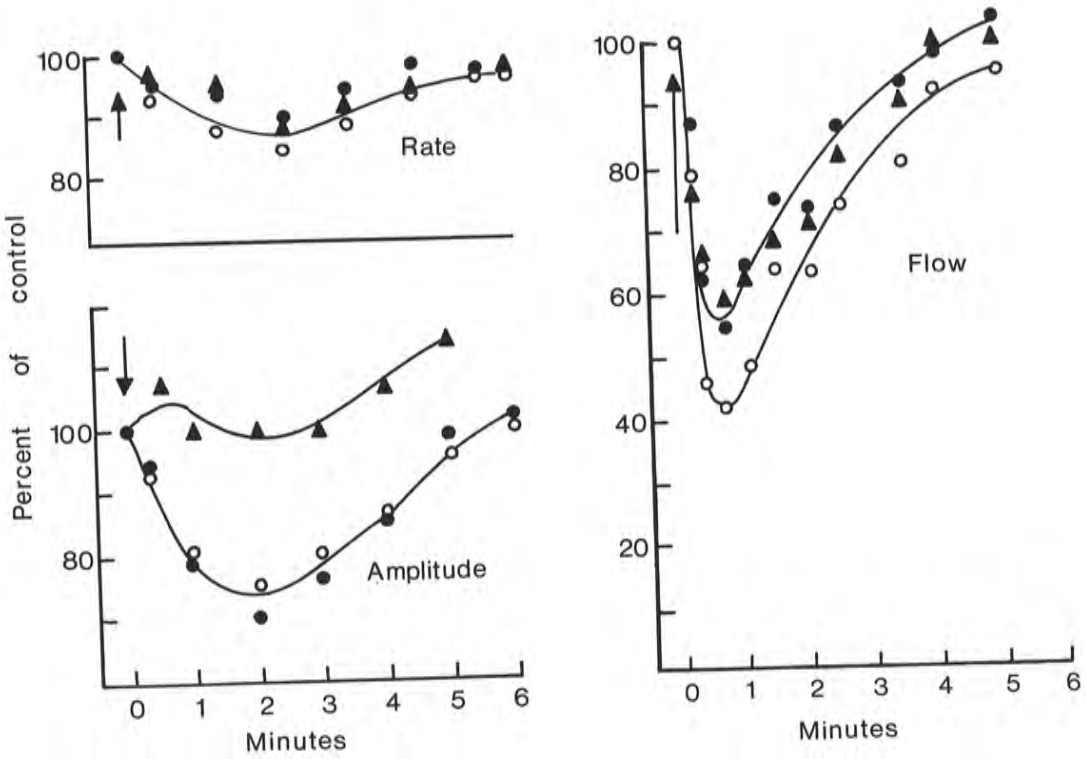


Figure 13: Effect of crude toxin on heart rate, amplitude of contraction and coronary flow. Three doses (each 0.03 M.U. added at arrow) separated by 15 minute rest periods have been administered to one heart. The variation in amplitude response shown is an extreme case. 0——0 first dose; ●——● second dose; ▲——▲ third dose.

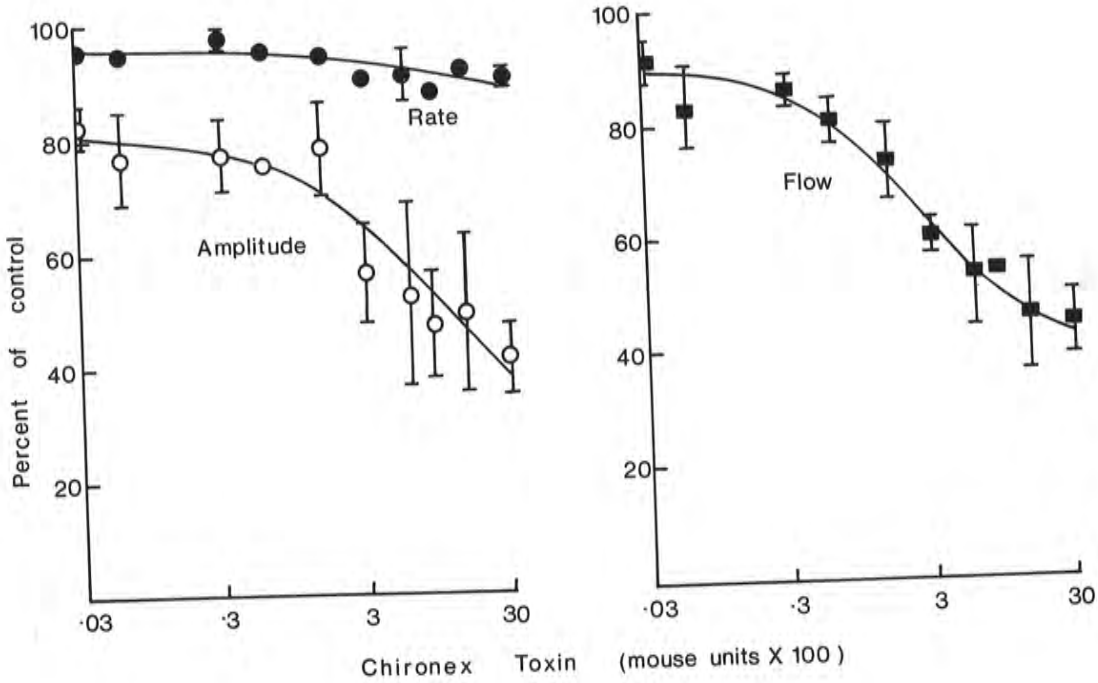


Figure 14: Effect of a range of doses of crude Chironex toxin on heart rate, amplitude of contraction and coronary flow. Semilogarithmic plot; the bars represent ± S.E. of mean of 4 - 9 observations. Where error bars are absent, the standard error was less than 1.5 per cent.

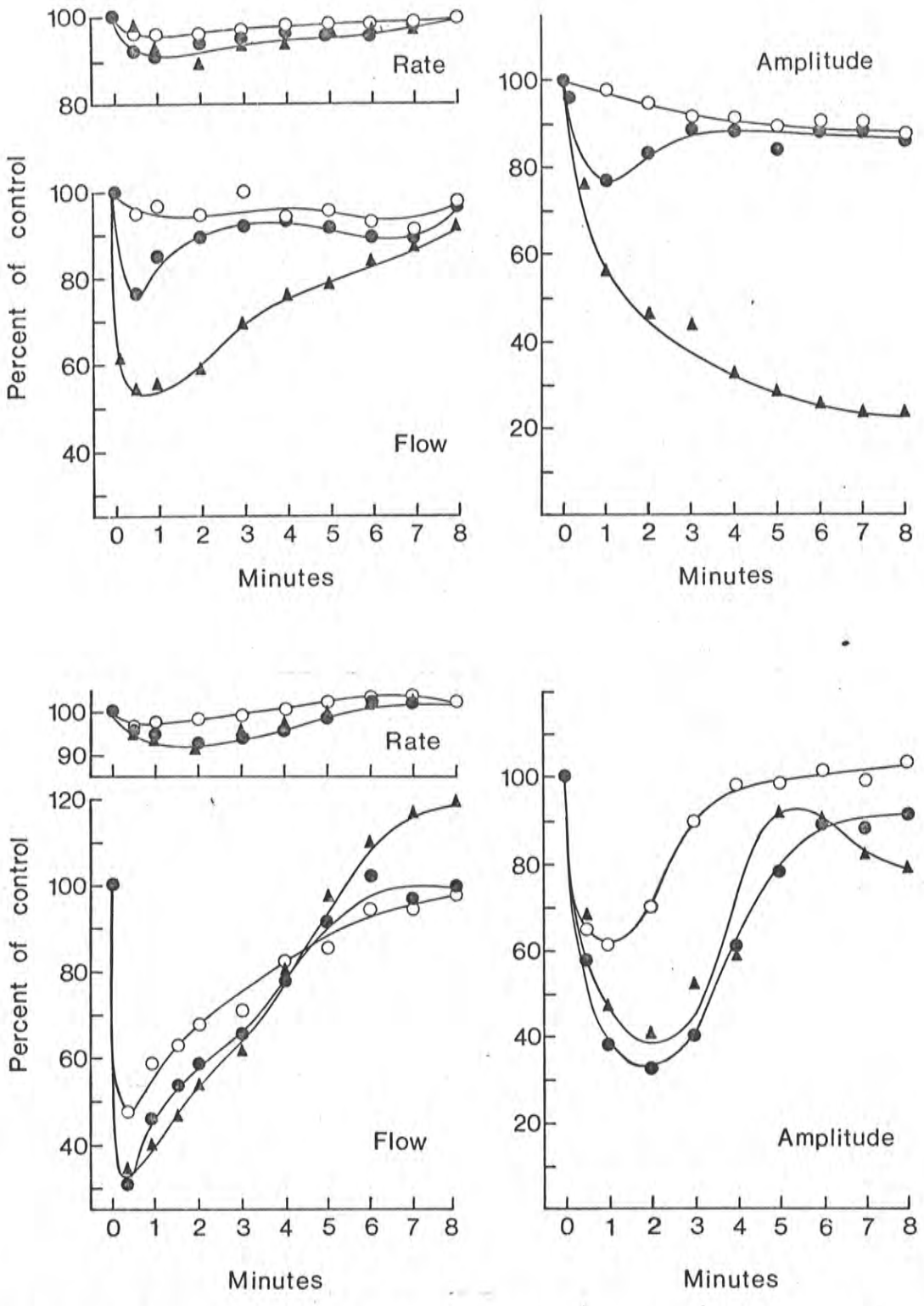


Figure 15: The effect of the haemolysin (upper) and cardiotoxin (lower) on rate, coronary flow and amplitude of contraction in the isolated perfused guinea pig heart. Doses are 0.03 (0—0), 0.3 (●—●) and 1.0 M.U. (▲—▲). Each curve is the mean of 3-6 observations.

rate and amplitude on the level of toxin was investigated for the crude toxin and both the Sephadex G-200 fractions. The percentage change in coronary flow was calculated using the control level over 2 - 3 minutes prior to addition of toxin and the minimum rate of flow obtained within 1 minute after the addition of the toxin. Rate and amplitude changes were calculated similarly except that the minimum rate and amplitude obtained within 3 minutes of the addition of the toxin was used. Table 5 details the percentage

Table 5. Effect of 0.03, 0.3 and 1.0 M.U. doses of Chironex toxins on the isolated heart. Figures shown are ± S.E.M. The number of observations is shown in parentheses.

		Crude toxin	Cardiotoxin	Haemolysin
	Dose (M.U.)			
Heart rate (% of control at 1 minute)	0.03	90.5±1.0 (9)	97.7±1.0 (5)	96.1±0.7 (6)
	0.3	90.0±1.5 (8)	93.8±1.0 (5)	91.2±2.1 (3)
	1.0	---	93.0±0.9 (4)	92.4±1.5 (6)
Coronary flow (% of control at 30 seconds)	0.03	60.5±3.0 (10)	55.0±4.4 (5)	95.8±3.7 (6)
	0.3	45.0±5.5 (9)	36.6±3.2 (5)	76.3±5.7 (3)
	1.0	---	34.1±6.0 (4)	54.6± 5.8 (6)
Amplitude of contraction (% of control at 1 minute)	0.03	56.5±8.5 (8)	60.6±12.6 (5)	96.7±4.0 (6)
	0.3	41.0±6.0 (8)	37.3±15.0 (5)	76.8±5.8 (3)
	1.0	---	46.5±16.8 (4)	56.5±15.4 (6)

changes in the three cardiac parameters after 0.03, 0.3 and 1.0 M.U. of crude toxin, cardiotoxin or haemolysin; dose response data are illustrated in figures 14 and 15. Doses of toxin were added at zero time in figure 15.

The dose-response curve of change in coronary flow against log dose of crude toxin is sigmoid over the range of concentrations employed; the decrease in heart rate was relatively constant. Amplitude response to low doses of toxin was similar to that obtained with coronary flow, but at higher levels there was no tendency for the curve to flatten; instead amplitude declined to zero. These effects are illustrated in figure 14. It can be seen from the standard error values (figure 14) that the change in amplitude is subject to increasing variation with increasing levels of toxin. Doses of toxin up to 0.3 M.U. evoked reversible changes in coronary flow, heart rate and amplitude of contraction. At or above this dose, these changes became partially or completely irreversible.

The response to cardiotoxin perfusion was similar; changes in coronary flow were perhaps even more transient, but amplitude and rate responses were identical (see Table 5). The effects of doses up to 1 M.U. were usually reversible, although at this level contraction amplitude was irreversibly depressed in some preparations.

The standard dose (0.03 M.U.) of haemolysin had little effect on the perfused heart. However, as the dose was increased, prolonged effects on coronary flow and contraction amplitude resulted (see figure 15). Doses of 1 M.U. invariably caused a coronary vasoconstriction which lasted 4 - 5 minutes, with a concurrent decrease in contraction

amplitude as ventricular contractions became weaker and finally ceased. This effect was irreversible.

The effects of all the toxin preparations are consistent with those observed in the intact animal. Small doses of crude toxin or cardiotoxin produce greater decreases in the three cardiac parameters than equivalent doses of haemolysin, although all are capable of producing ventricular asystole in larger doses. It can be seen from these results that the crude toxin response corresponds to a combination of the effects of the cardiotoxin and the haemolysin. The cardiotoxin produces the rapid, transient changes which predominate after small doses of crude toxin, whereas the irreversible elements of the haemolysin response are increasingly evident as the dose of toxin is increased.

It is possible that the coronary vasoconstriction produced by the toxin may result in some degree of hypoxia in the isolated heart. This in turn could cause the observed decrease in contraction amplitude. Experiments were therefore conducted in which the heart was perfused under constant flow rather than constant pressure conditions. The pump and recording system were the same as those used in the isolated rabbit ear preparations. Flow rate was 10 ml per minute.

Doses of 0.03 or 0.3 M.U. of the cardiotoxin increased coronary perfusion pressure to 160 - 170 per cent of the control value (48mm Hg). The increase was shortlived and perfusion pressure returned to normal within 4 - 6 minutes. Decreases in heart rate were similar to those observed previously. The amplitude of contraction was

also decreased, although the decrease was only 50 per cent of that observed in the constant pressure experiments. The shorter time of exposure to the toxin under constant flow conditions may account for this difference.

The marked ability of the toxin to reduce coronary flow prompted an investigation of the effects of several vasodilator agents on this response. Crude toxin extracts were used in all of these experiments.

Effects of Chironex toxin in the presence of catecholamines.

When adrenaline (0.1 µg/ml), noradrenaline (0.1 µg/ml) or isoprenaline (0.1 µg/ml) was added to the perfusing fluid, several changes were observed. Coronary flow was increased, the most marked rise being produced by isoprenaline which increased flow to 160 per cent of the control level; adrenaline was less effective and noradrenaline was least effective. Heart rate was increased to the same extent by each compound and amplitude of contraction was increased equally by adrenaline and isoprenaline. Noradrenaline had little augmenting effect on amplitude and in some preparations the amplitude was decreased. The effects of catecholamines are consistent with those obtained by other workers (Satchell et al., 1968; Glomstein et al., 1967).

The response to 0.1 µg/ml adrenaline is illustrated in figure 16; adrenaline was added at zero time in each case. For each of the catecholamines, the maximum response was obtained after two minutes perfusion and remained constant over the next ten minutes. This permitted the toxin to be added after two minutes and the course of its action to be followed in the presence of the catecholamine.

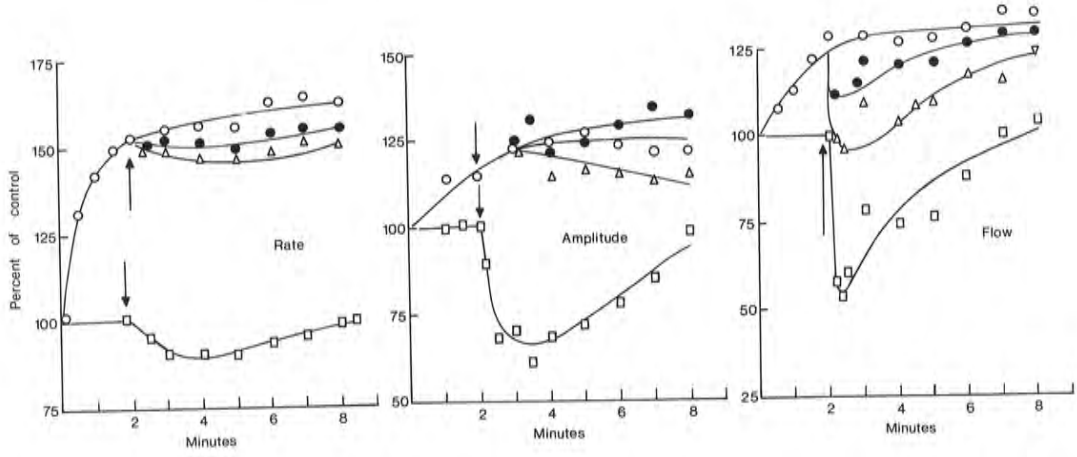


Figure 16: Effect of a standard dose of crude toxin in the presence of 0.1 µg/ml adrenaline. Adrenaline is added at zero time; toxin is added at the arrow. Percentage changes after addition of toxin in the presence of adrenaline are calculated using the heart rate, amplitude and coronary flow values observed 2 minutes after addition of adrenaline as 100 per cent values. □ —□ 0.03 M.U. toxin, normal perfusate; 0—0 0.1 ug/ml adrenaline added to perfusion fluid at zero time; ● —● 0.03 M.U. toxin in presence of adrenaline; △ —△ 0.15 M.U. toxin in presence of adrenaline.

Control responses in the absence of catecholamines were obtained before and after this procedure.

The effect of Chironex toxin in the presence of adrenaline is illustrated in figure 16. The chronotropic response to the standard toxin dose (0.03 M.U.) under control conditions was small and it was not possible to say whether adrenaline had a significant effect on this change. However, adrenaline had a substantial influence on the changes in coronary flow and contraction amplitude which follow toxin administration. The standard toxin dose administered in the presence of adrenaline produced no significant change in the amplitude and even five times the normal dose of toxin had little effect. The coronary flow in the presence of adrenaline was decreased only slightly by the normal toxin dose and five times this level (0.15 M.U.) was required to approach the flow changes observed in the control.

Toxin administered in the presence of 0.1 µg/ml isoprenaline produced similar effects. Decrease in coronary flow by the standard toxin dose was insignificant and even at five times this dose the decrease was only about one-third of that observed in a standard control dose. Changes in rate and amplitude were similar to those observed in the presence of adrenaline. Administration of the toxin in the presence of noradrenaline produced similar results.

Effect of Chironex toxin in the presence of alpha or beta adrenergic blocking drugs.

As perfusion of catecholamines modified the response to the toxin, experiments were conducted to determine the

effect of toxin on a heart in which either the alpha or beta adrenergic receptors were blocked by phentolamine or propranolol respectively.

Phentolamine (1 µg/ml) and propranolol (1 - 2 µg/ml) both decreased the heart rate slightly and had little or no effect on coronary flow. Amplitude of contraction, however, showed a slow decline over the period of perfusion, but returned to normal levels when normal nutrient fluid was restored.

The effect of the toxin on coronary flow, heart rate and amplitude of contraction was not significantly modified by phentolamine at the level used. Propranolol had no effect on amplitude or rate responses to the toxin, but the decrease in coronary flow was consistently reduced by a small amount when the toxin was injected in the presence of the beta blocking agent.

Effects of Chironex toxin in the presence of 5-hydroxytryptamine.

The addition of 2 µg/ml 5-hydroxytryptamine (5-HT) to the perfusion fluid produced increases in coronary flow, heart rate and amplitude of contraction. Coronary flow was increased by 34 per cent, heart rate was increased by 19 per cent and amplitude of contraction by 9 per cent (all increases are means of four observations). The responses reached a maximum within 2 minutes and then remained constant over the next 10 minutes.

Coronary flow and heart rate responses to Chironex toxin in the presence of 5-HT were not significantly different from those obtained as controls in the absence of

5-HT. The toxin appeared to have a greater effect on amplitude in the presence of 5-HT than in the control situation but the difference was not great. The results are summarized in Table 6.

Table 6. Changes produced in the three heart parameters after 0.03 M.U. Chironex toxin in the presence and absence of 2 µg/ml 5-HT. Results are the mean of two experiments.

	Heart rate (beats/min)	Amplitude of contraction (mm)	Coronary flow (ml/min)
Control	170	21	7.4
Control after toxin	150	17.5	4.0
2 µg/ml 5-HT	200	22	9.9
5-HT and toxin	180	17	5.3

Effects of Chironex toxin in the presence of adenosine and adenosine triphosphate (ATP).

Adenosine and ATP are both potent coronary vasodilators (Wedd, 1931; Rowe et al., 1962; Rowe, 1968). ATP was found to be more effective in this series of experiments. The changes in coronary flow, heart rate and amplitude produced by 0.4 µg/ml adenosine are shown in figure 17. 0.8 µg/ml ATP had similarly small effects on heart rate (increased by 10 per cent, mean of 4 observations) and amplitude of contraction (increased by 18 per cent). The effect of ATP on coronary flow, however, was considerable; the flow was increased from the mean control level of 8.1 ml

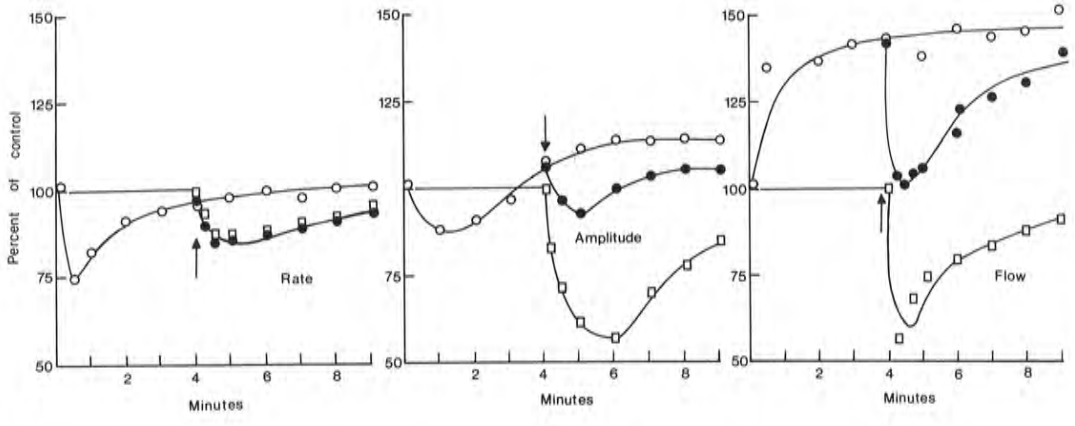


Figure 17: Effect of a standard dose of crude toxin in the presence of 0.4 µg/ml adenosine. Adenosine is added at zero time; toxin is added at arrow. Percentage changes after addition of toxin in the presence of adenosine are calculated using the heart rate, amplitude and coronary flow values observed 4 minutes after addition of adenosine as 100 per cent values. □——□ 0.03 M.U. crude Chironex toxin, normal perfusate; 0——0 0.4 ug/ml adenosine added to perfusion fluid at zero time; ●——● 0.03 M.U. toxin in presence of adenosine.

per minute to 16.2 ml per minute.

Both adenosine and ATP produced an initial transient decrease in heart rate and amplitude of contraction. This effect is shown for adenosine in figure 17. The onset of response to adenosine and ATP was slower than for the other vasodilators, taking 3 - 4 minutes to reach a maximum level. Chironex toxin in these experiments was therefore added 4 minutes after the vasodilator. The effect of the toxin in the presence of adenosine is illustrated in figure 17. The reduction in coronary flow and heart rate was not significantly altered by the presence of either adenosine or ATP although the decrease in amplitude was reduced in both cases. In the presence of ATP the reduction in amplitude after the toxin was 12 per cent; without ATP the reduction was 45 per cent. Thus, adenosine and ATP can partially antagonize the negative inotropic effect of the toxin, but are relatively ineffective antagonists of the negative chronotropic effect and the coronary vasoconstriction.

Investigation of the effects of the toxin fractions on vascular smooth muscle was extended by the use of several isolated blood vessel preparations.

PERFUSED RABBIT EAR

Injection of Chironex cardiotoxin into the perfusion system produced a constrictor effect on the ear vasculature. The constriction was dose-dependent between 0.1 M.U. and 5 M.U. Doses greater than 5 M.U. produced no greater effect. Response to a fixed dose of cardiotoxin was reproducible, provided sufficient time was left between doses. Perfusion

pressure reached 200mm Hg in some preparations at the peak of the cardiotoxin response; this was approximately the same as the peak response to noradrenaline. The toxin response was more prolonged than control responses to histamine or noradrenaline.

Doses of cardiotoxin up to 15 M.U. had no significant effect on the noradrenaline dose-response curve. Neither the alpha-blocking agent, phentolamine (1 µg/ml), nor the beta-blocking drug, propranolol (10 µg/ml), had any effect on the toxin response.

Injection of doses of Chironex haemolysin greater than 0.5 M.U. produced a constriction of the ear vessels. Responses tended to be more prolonged than those observed after the cardiotoxin and the maximum response was only 50 - 60 per cent of the peak cardiotoxin response. Doses of haemolysin greater than 0.5 M.U. depressed the response of the preparation to noradrenaline but the histamine response did not appear to be affected.

Rabbit Ear Artery.

Injection of either cardiotoxin or haemolysin into the perfusion system was followed by a 30 second delay in perfusion to allow time for diffusion of the toxin through the intima. The perfusion was then restored in order to record the response to the toxin. Control experiments had shown that the response to noradrenaline was not affected by the 30 second delay period.

The artery was constricted by doses of cardiotoxin greater than 0.1 M.U. Maximum response (75mm Hg) occurred at doses of 5 M.U.; this was less than half of the maximum noradrenaline constriction. The cardiotoxin had no

observable effect on the response of the preparation to noradrenaline.

The haemolysin produced a constriction of the artery in doses greater than 0.1 M.U. The response was approximately the same as the cardiotoxin response. Doses of haemolysin in excess of 1 M.U. depressed the response of the preparation to noradrenaline by a variable amount.

Rabbit Ear Vein.

Investigations of the response of the isolated ear vein to the toxin were hindered by the rapid development of tachyphylaxis which occurred in this preparation. Doses of cardiotoxin between 0.1 M.U. and 10 M.U. produced a constriction of the vein, however the effect of cardiotoxin on histamine or noradrenaline responses could not be determined because responses could not be duplicated. Doses of haemolysin from 0.1 M.U. to 100 M.U. produced no observable effect.

RABBIT AORTA

After a latency of some minutes 0.05 - 1.0 M.U. per ml cardiotoxin produced slow reversible contractions of the helical aortic strip. The response of the preparation to noradrenaline or histamine was unaffected by any of the doses of cardiotoxin used.

0.2 M.U. per ml haemolysin produced a similar delayed contraction of the rabbit aorta preparation. After exposure to this level of haemolysin, the sensitivity of the preparation to noradrenaline was reduced one hundred fold; histamine sensitivity was decreased by a slight amount.

DISCUSSION

The similarity of the pharmacological effects produced by tentacle extract and amnion 'milked' toxin suggests that the same toxic principles are present in both preparations. This view is supported by the work by Crone and Keen (1970) in which the same two fractions have been obtained from each of these toxin preparations by cation exchange Sephadex chromatography. The possibility that Sephadex chromatography might remove pharmacologically active substances of low molecular weight has been covered by the use of amnion 'milked' toxin and tentacle extract which had not been chromatographed. Substances such as 5-hydroxytryptamine and tetramine, which are present in many species of Cnidaria, appear to be either absent or present in insignificant amounts.

There is considerable evidence to suggest that the toxic principles are protein in nature. They are extremely potent, high molecular weight compounds which are highly labile at room temperature. In addition, antisera which offer some degree of protection against lethal, haemolytic and dermatonecrotic effects have been produced (Keen, 1970; Baxter et al., 1968). The activity of the haemolysin has been shown to be dependent on pH and temperature, and further studies have shown that the haemolysin can be chromatographed on both cation and anion exchange resins, suggesting that it has an isoelectric point near neutrality (Crone and Keen, 1969). No other class of compound could exhibit all of these properties, although it remains possible

that the biological activity of the compound could be due to a prosthetic group attached to the main protein molecule.

The toxin is rapidly lethal only when injected directly into the blood circulation; many times the intravenous lethal dose are required for intraperitoneal or subcutaneous administration to be lethal. It is possible that death after administration of the toxin by these routes is dependent upon the introduction of the toxin into the blood circulation in the region of the injection.

The toxin preparations which have been isolated from Chironex fleckeri have a number of properties in common. They are all cardiotoxic and death following injection appears to be due to respiratory arrest and cardiovascular failure. Respiratory arrest is central in origin, as is shown by the results illustrated in figure 11 and the finding that stimulation of the phrenic nerve post mortem produced a strong diaphragmatic twitch.

The initial hypertensive response observed in anaesthetized animals is produced by a direct vasoconstriction. The brief duration of this action on the isolated heart and the isolated blood vessel preparations suggests that the effect on the systemic circulation may also be transient.

After the initial hypertensive period, there is a rapid fall in blood pressure followed by one or more pressure oscillations. This response has also been reported by Endean et al., (1969) after injection of toxin from washed nematocysts into anaesthetized rats. Initially, the fall in blood pressure was attributed to the direct effect of the toxin on the heart, however, investigation of the

effects of the Sephadex G-200 fractions on vasomotor reflexes suggested that the toxins may further modify the cardiovascular response by baroreceptor stimulation and/or depression of the brain stem vasomotor centre. The profound blood pressure oscillations seen after administration of the toxins suggest a disordered feedback system such as could occur after interference with vasomotor reflexes.

Thus, the hypotension which follows the initial arterial pressure rise may result from a combination of baroreceptor stimulation, which would reduce vasomotor tone, and a fall in cardiac output. The subsequent apnoea would cause stimulation of peripheral and central chemoreceptors, and thus increase the efferent vasomotor outflow. Arterial pressure would then increase until restitution of respirations caused a reduction in chemoreceptor drive; arterial pressure would then decrease once again. Thus blood pressure oscillations could continue until either the heart or the CNS ceases to function effectively.

Evidence for the involvement of some part of the baroreceptor reflex arc is supplied by the observation that the carotid occlusion reflex was depressed at a time when vasoconstriction by noradrenaline was demonstrable. This was observed in both rats and rabbits after either the cardiotoxin or the haemolysin. The finding is particularly relevant in the rabbit, where the reflex could not be elicited at a time when the arterial pressure was nearly normal. Thus, the reflex cannot have been suppressed by hypotension alone.

The occurrence of arterial pressure oscillations

after baro- and chemo-receptor denervation is consistent with the suggested mode of action of Chironex toxin on the cardiovascular system. The first fall in blood pressure, which, in these denervated animals would result solely from a fall in cardiac output, was slower than usual, and the pressure oscillations were in phase with the periods of apnoea rather than out of phase as in the intact animal. Peripheral chemoreceptor stimulation due to hypercapnia appears to raise the blood pressure before stimulation of the respiratory centre leads to restitution of respirations. However, when these chemoreceptors are denervated the blood pressure rise and the return of respirations are synchronized.

Although these experiments do not conclusively differentiate between baroreceptor stimulation and depression of the vasomotor centre, the large size of the toxin molecules favours a peripheral action.

Interference with the efferent arm of the reflex arc by blockade of ganglionic transmission (hexamethonium), adrenergic nerve transmission (bretylium) or the alpha adrenergic receptor (phenoxybenzamine) either greatly diminished or abolished the blood pressure oscillations. This would be expected because the opposing effects of baro- and chemo-receptor stimulation would be ineffective and the cardiovascular response would result simply from transient vasoconstriction and cardiac depression.

The clear-cut relationship between the arterial pressure oscillations and the periods of apnoea, which was seen after the Sephadex G-200 toxin fractions, was obscured when a crude toxin preparation was used. The slightly

different rates of onset of the effects of the two toxins suggests that their combined injection during envenomation will cause symptoms due to interactions between these effects.

It is not possible to assess the relative importance of these factors in determining the human response to the toxin. However, the great development of vasomotor reflexes in man, because of his erect posture, could indicate that baroreceptor stimulation plays an important part in the action of the toxins. Barnes (personal communication) has noted that victims of Chironex stings frequently 'rally' just prior to death. This may correspond to the situation in the experimental animal.

The finding that the toxin is haemolytic confirms the observation of Weiner reported by Southcott and Kingston (1959). Investigations by Keen and Crone (1969a) have shown that the haemolysin is labile at room temperatures. The rate of haemolysis was found to be dependent on temperature, pH and the dilution of the extract. Haemolytic activity was inhibited by sucrose or plasma and accelerated by benzene; cholesterol or lecithin had no effect. The susceptibility of red cells to haemolysis was found to vary from one species to another. Of the species tested, guinea pig erythrocytes were least sensitive and human and rat erythrocytes most sensitive.

The haemolytic activity is an integral part of the activity of the whole toxin, although the associated rise in plasma K^+ observed in the anaesthetized animal experiments was not consistently high enough to be causally related either to death or to the cardiac irregularities.

Intravascular haemolysis has not been described in humans after Chironex stingings and it is unlikely that this condition and the associated haematuria would remain unobserved. The absence of this effect may be due in some cases to inactivation of the haemolysin at body temperature by plasma; in others death may follow so rapidly that haemolysis has little or no time to occur.

It is possible that other manifestations of the toxin may be caused by alterations in membrane permeability. Such an effect could be responsible for the pain and wealing experienced by victims of Chironex stingings. Potassium accumulation at nerve endings in the skin produces pain (Keele and Armstrong, 1964); changes in capillary permeability may be responsible for oedema. Investigations of the dermatonecrotic activity by Keen and Crone (1969b) indicate that, in rats and guinea pigs, necrosis is the result of a direct effect on the cells of the epidermis and dermis. Although there is capillary damage with thrombosis within the area of damage, there appears to be only a mild vascular response around the lesion. The authors suggest that the toxin acts directly on the cell membrane in a manner similar to that which produces haemolysis, resulting in rapid death of the cell. Antihistamines and 5-HT antagonists had no effect on the development of the lesion. Experiments with the toxin fractions produced by Sephadex G-200 chromatography showed that only the haemolysin possesses dermatonecrotic activity.

The perfused heart and blood vessel preparations have been used so that the cardiovascular effects of Chironex toxin preparations could be examined further. Although

the heart preparation is somewhat artificial in nature (Opie, 1965) the results showed a reasonable correlation with those observed in the intact animal. Bradycardia followed a similar time course in both preparations and high levels of toxin resulted in irregular rhythms in both intact and isolated hearts. The reduction in amplitude of contraction in the isolated heart is consistent with the decrease in cardiac output which occurs in the whole animal.

The coronary vasoconstriction, demonstrated by decreased coronary flow or increased coronary perfusion pressure, which is produced by Chironex toxin in the isolated heart, is of considerable interest. If this occurs in the intact animal it would combine with the poor venous return and inadequate ventilation to cause hypoxic changes in the myocardium. In the isolated heart, the coronary vasoconstriction produced by the standard dose of toxin (0.03 M.U.) is too brief to cause a significant degree of hypoxia; however, at doses comparable to those which are lethal in the whole animal, the constriction is more prolonged and could reasonably influence the rate and amplitude changes.

It is interesting to note that all of these effects occur in the isolated heart in the absence of neural control; this is in agreement with the concept that the toxin has a direct cardiotoxic effect in the intact animal.

The vasodilator drugs were employed in an attempt to counteract the toxic response and in particular to reduce the coronary constriction. Coronary flow is increased by catecholamines either by a direct effect on the coronary vessels or by a secondary effect due to stimulation of

cardiac metabolism (Glomstein et al., 1967; Rowe, 1968); adenosine and ATP produce coronary vasodilatation by a direct effect on either the vasomotor nerves or the vessel wall (Rowe et al., 1962). The mechanism of action of 5-hydroxytryptamine has not been clarified (Rowe, 1968). All of these compounds increased coronary flow in the control situation but only the catecholamines antagonized the flow rate changes produced by the toxin.

The decrease in coronary flow produced by Chironex toxin is most likely to be a direct constrictor effect on the coronary vessels. The ineffectiveness of adenosine and ATP in antagonizing the coronary constriction may be due to a different site of action of these compounds and Chironex toxin; adenosine and ATP produce dilatation at the capillary level (Rowe et al., 1962; Rowe, 1968) while Chironex toxin and the catecholamines may both act at the arterial or arteriolar level. The toxin, however, does not appear to affect either alpha or beta adrenergic receptors as prior blockade of these produced no significant change in the toxin response.

The cause of the decrease in contraction amplitude and heart rate produced by the toxin cannot be well defined from these experiments. With large toxin doses hypoxia is likely to contribute to these changes, but their occurrence at low toxin doses where coronary vasoconstriction is too brief to produce significant hypoxia requires the presence of another factor. It may be suggested that the lytic properties of the toxin, which are considered to be responsible for the haemolysis, dermatonecrosis and possibly the action on the trachea and ileum preparations, may also

affect the conducting system of the heart and ventricular contractility. This would explain the results observed after administration of all the toxin preparations with the exception of the cardiotoxic fraction from Sephadex G-200 chromatography which is neither haemolytic nor dermatonecrotic. The mode of action of the cardiotoxin on heart rate and amplitude of contraction must remain unexplained at the present time. The improvement in amplitude of contraction produced by adenosine, ATP and the catecholamines in the presence of the toxin suggests that these compounds may improve the metabolic status of the intoxicated heart.

Experiments by Keen (1970), in which 2.5 µg/kg adrenaline was given intravenously to anaesthetized rats immediately after the administration of a known lethal dose of toxin, could not demonstrate any protective effect due to the presence of the catecholamine. However, this finding is not totally unexpected as coronary vasoconstriction is only one of a number of effects of the toxin which may combine to result in a lethal outcome.

Although the experiments with Chironex toxin preparations have described the various changes which lead to death in the anaesthetized animal, as yet little progress has been made towards the development of an effective treatment for human victims. Even though an antivenin has been produced, its use in the treatment of lethal stingings must be restricted by the difficulty of reaching victims sufficiently early for administration to be of any value. The antivenin appears to be effective in reducing the dermatonecrosis associated with non-lethal stingings in

humans (J.H. Barnes, personal communication) but the number of cases treated is too small to draw any positive conclusions.

At this stage, it would appear that the prevention of stingings remains the only sure method of avoiding human fatalities.

THE PHARMACOLOGICAL PROPERTIES OF OCTOPUS MACULOSUS TOXIN (MACULOTOXIN)

Octopus maculosus has been responsible for a number of cases of temporary paralysis among people who have handled the animal on Australian beaches. Victims have described similar symptoms, which include weakness, nausea and respiratory difficulty. Three fatalities have occurred and death has been ascribed to respiratory failure due to flaccid paralysis.

The species of octopus appears to be moderately abundant in Australian coastal waters during the summer months. It is not, however, a serious hazard to swimmers since it is not aggressive, and has a very small 'beak', which would make injection of venom into a human victim difficult. As victims are usually unaware of the envenomation until symptoms develop, it is possible that bites occurring underwater may go unnoticed as the venom could wash out of a shallow wound. The venom is secreted by the large posterior salivary glands of the octopus and is used to immobilize the small crabs that are its prey.

Preliminary studies on the properties of the toxin have been published by Simon et al., (1964) and the aim of the present study has been to investigate further the biological properties of the toxin. It became evident during this work that maculotoxin is pharmacologically very similar to tetrodotoxin and saxitoxin and in several experiments responses to maculotoxin have been compared with responses to these compounds.

RESULTS

EFFECTS ON MICE

Following intravenous injection of maculotoxin mice became agitated, ataxic, and the hind limbs splayed out and became paralyzed. Respirations were laboured and ceased after 60 - 90 seconds; there were brief but violent terminal convulsions. At autopsy the heart was found to be beating irregularly. Deaths in mice after tetrodotoxin followed an identical pattern to those seen after maculotoxin.

The relationship between the minimum lethal dose by intravenous injection and the intraperitoneal 'mouse unit' as calculated by workers with other neurotoxins may be seen from figure 18, in which the logarithm of the intravenous dose is plotted against the reciprocal of the time to death after intraperitoneal injection. Data for both maculotoxin and tetrodotoxin are shown. If the intraperitoneal 'mouse unit' (M.U.) is defined as the dose required to kill a mouse in 10 minutes after i-p injection (Kao, 1966), then approximately 1.8 intravenous M.U. are equivalent to 1 intraperitoneal M.U. Data in the present study are expressed in terms of the intravenous unit.

It can be seen that the time to death data for maculotoxin and tetrodotoxin, illustrated in figure 18, are coincident, whereas Konosu et al., (1968) have shown that the time to death data for saxitoxin yield a significantly different slope from the tetrodotoxin data. Therefore, in this respect, maculotoxin appears to resemble tetrodotoxin more closely than saxitoxin.

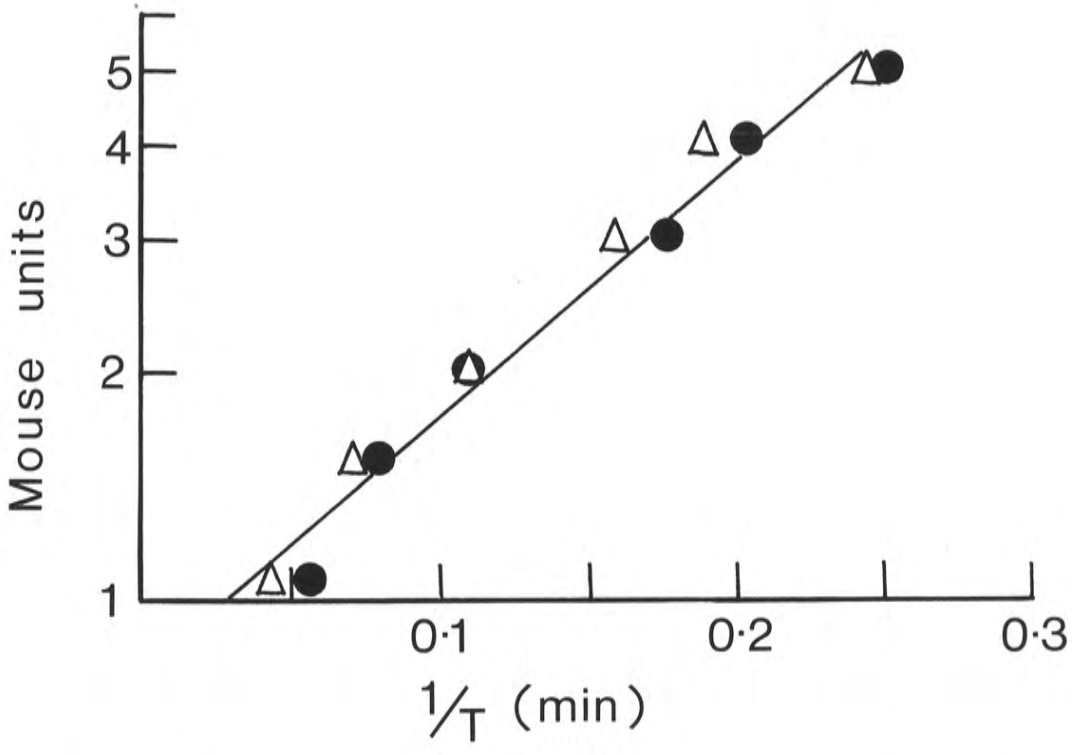


Figure 18: Relationship of dose and time to death after maculotoxin and tetrodotoxin. The logarithm of dose (intravenous M.U.) is plotted against the reciprocal of the time to death (minutes), following intraperitoneal injection. Maculotoxin (Δ-Δ); tetrodotoxin (●-●). 6 - 8 mice were used at each dose level.

EFFECTS ON ANAESTHETIZED ANIMALS

Arterial and venous pressure, respiratory rate and depth and electrocardiographs were recorded. Anaesthetized rabbits proved to be very sensitive to the toxin; intravenous doses of 3 - 8 M.U./kg were sufficient to cause hypotension, bradycardia and respiratory depression. Rats were less sensitive; intravenous doses of approximately 15 M.U./kg were required to produce similar depressant effects.

These non-lethal doses lowered the systolic arterial pressure by up to fifty per cent within three minutes of injection. Venous pressure was usually unchanged in both species although some rats showed a slight increase in right atrial pressure as the arterial pressure fell. Pulse pressure was not altered. The heart rate was reduced over the same period and ECG traces were unchanged except for the bradycardia.

Rats occasionally showed a transient increase in respiratory amplitude after toxin injection, however this was shortlived, and in both species respiratory amplitude declined during the period of hypotension. The respiratory depression was more transient than the cardiovascular effects.

The changes produced by non-lethal doses of maculotoxin in rabbits and rats are tabulated in table 7. Cardiovascular effects persisted for 20 - 30 minutes in the rabbit and slightly longer in the rat.

The toxin appeared to have a cumulative effect, since a second dose of similar magnitude administered to

Table 7. Maximum changes in cardiovascular and respiratory parameters within 4 minutes after intravenous injection of non-lethal doses of maculotoxin into rabbits and rats. Values are ± S.E.M., n is the number of experiments.

		Systolic blood pressure (mm Hg)	Heart rate (min^{-1})	Respiration rate (min^{-1})
Rabbit (n=5)	Control	122±6	300±17	58±2
	Toxin	58±3	250±10	36±3
Rat (n=5)	Control	158±8	398±7	68±1
	Toxin	95±7	302±12	62±2

a rabbit after all symptoms had subsided was usually sufficient to cause respiratory failure (see also Simon et al., 1964). A second dose of 24 - 40 M.U./kg toxin brought about respiratory arrest in the rat in 1 - 4 minutes. The rate and amplitude of respirations was decreased immediately after injection of a lethal dose of toxin. When death was prolonged over several minutes, respirations became very slow, prolonged and gasping and occasionally a period of apnoea preceded a final respiratory effort. The arterial pressure decreased as these changes progressed and ranged from 30 - 50 mm Hg when respirations ceased.

Venous pressure increased as arterial pressure fell, and the ECG showed marked bradycardia and T wave enlargement.

The observation that hypotension after maculotoxin injection in both the rabbit and the rat always accompanied or preceded respiratory depression suggests a closer similarity with tetrodotoxin than with saxitoxin (Kao and Nishiyama, 1965).

Effect of catecholamines on response to toxin

In both rabbits and rats hypotension due to a non-lethal dose of toxin was temporarily reversed by adrenaline or noradrenaline. Experiments were designed so that a test dose of catecholamine was given before and after a non-lethal dose of toxin.

In the rat 1.5 µg/kg l-noradrenaline bitartrate raised the systolic arterial pressure to approximately 200 mm Hg, without changing heart rate or venous pressure. A similar dose administered 5 minutes after 15 M.U./kg maculotoxin also raised the systolic arterial pressure to 200 mm Hg, although the arterial pressure had been reduced by maculotoxin. Heart rate and venous pressure were again unchanged.

The arterial pressure was increased by a similar amount by l-adrenaline bitartrate (2 µg/kg) without change in heart rate but with a slight increase in venous pressure. After treatment with maculotoxin, adrenaline increased the depressed arterial pressure to the same level, but in this instance there was a significant increase in heart rate. Comparable experiments, in which tetrodotoxin was substituted for MTX, produced very similar results.

Rabbits responded to doses of 2.5 µg/kg noradrenaline and 7 µg/kg adrenaline in the same way. These results can be compared with those of Kao and Fuhrman (1963).

Since the terminal fall in arterial pressure is precipitous and is likely to contribute to the fatal outcome, noradrenaline (2.5 µg/kg) was given to rabbits during the period of respiratory depression which preceded terminal apnoea. However, this did not prevent the terminal fall in arterial pressure.

Effect of maculotoxin on phrenic nerve activity

The respiratory depression produced by maculotoxin was further examined by monitoring the electrical activity in the phrenic nerve and the diaphragm muscle in the rabbit. The results of one such experiment are shown in figure 19.

The anaesthetized animal had a respiratory rate of 50 per minute and an arterial pressure of 120 mm Hg. Inspiration lasted 500 msec and the respiratory pause was 700 msec. After maculotoxin (5 M.U./kg), the arterial pressure was reduced to 60 mm Hg and the respiratory rate to 35 per minute. Diaphragmatic activity lasted 1.2 seconds and the respiratory pause was 500 msec. The animal appeared to be adequately ventilated, and during the next 15 minutes the arterial pressure rose to 105 mm Hg and the respiratory rate returned to the control level. A second i-v injection of 5 M.U./kg MTX was given at this time. This produced a fall in arterial pressure of 45 mm Hg and respirations were reduced to approximately 30 per minute. The diaphragm showed almost continuous electrical activity, although both amplitude and frequency of the muscle potentials were somewhat

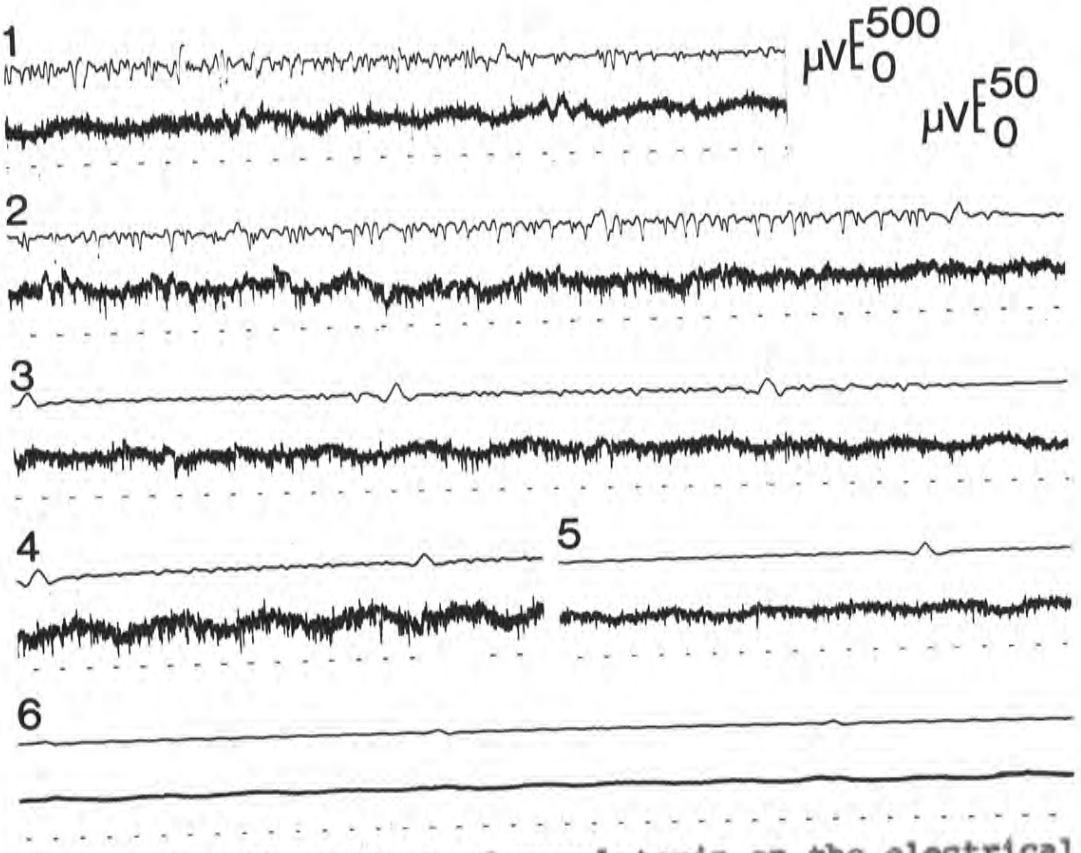


Figure 19: The effect of maculotoxin on the electrical activity of the phrenic nerve and diaphragm. Panel 1, part of a control respiration; Panel 2, a prolonged respiration after 5 M.U./kg maculotoxin; Panel 3, diminished diaphragmatic activity after a further 7 M.U./kg; Panel 4, increased phrenic activity which had little effect on the diaphragm; the ECG shows clearly; Panel 5, the onset of failure of phrenic volleys; Panel 6, electrical silence at death. Time marker = 0.02 seconds.

reduced (see figure 19, panel 2). There was a concurrent increase in amplitude of phrenic volleys. However, the animal survived, and a final dose of 7 M.U./kg was necessary to abolish respirations; apnoea occurred three minutes after this injection. Initially, breathing became largely intercostal and a decline in the amplitude of the myograph output was observed. The phrenic volleys increased in amplitude, although they produced only a ripple on the myograph trace. Finally, the phrenic impulses also diminished in frequency and amplitude until at death there was electrical silence. These effects are illustrated in figure 19, panels 3 - 6. After respirations ceased a tetanic stimulus applied to the phrenic nerve produced a weak diaphragmatic contraction.

Resuscitation of animals after maculotoxin

As the cause of death after maculotoxin envenomation appears to be respiratory paralysis, associated with profound hypotension, attempts were made to resuscitate rats and rabbits by positive pressure ventilation with a Palmer respiration pump.

It was possible to revive animals only after marginally lethal doses of MTX or TTX, and then only if the pump was started before respiratory depression became severe. Animals which were breathing but cyanotic, and which had a systolic blood pressure of less than 40 mm Hg, had a poor prognosis. In some cases, arterial pressure increased to approximately 60 mm Hg and the ECG returned to a near normal pattern during artificial ventilation, but spontaneous respiration did not occur even after ventilation

of the animal for up to two hours. When successful, artificial ventilation had to be continued for more than an hour after the return of spontaneous respiratory efforts in order to maintain the animal. This slow recovery of the paralyzed musculature is consistent with the slow return of activity in the isolated diaphragm preparation after exposure to maculotoxin.

EFFECTS ON ISOLATED ORGAN PREPARATIONS

Rat phrenic nerve-diaphragm

At concentrations equal to or greater than 0.15 M.U. per ml. maculotoxin brought about complete blockade of the isolated phrenic nerve-diaphragm preparation. The muscle remained responsive to direct stimulation after transmission was blocked, but the threshold was raised, a 5 - 10 fold increase in stimulating voltage being required for development of maximum tension. Large doses or prolonged treatment rendered the muscle inexcitable. Shortly before transmission failed the preparation was able to sustain a 10 second tetanus. The rate of onset of blockade was independent of the frequency of nerve stimulation over the range 6 - 60 per minute.

Neuromuscular blockade was characterized by a long latent period when twitch tension declined very little, followed by a rapid decline in tension which followed an exponential time course. Figure 20 illustrates the onset of blockade. Kao and Nishiyama (1965) observed a similar latency when measuring the decline in endplate potential after saxitoxin treatment. Recovery was prolonged; it was necessary to wash preparations for upwards of 90 minutes

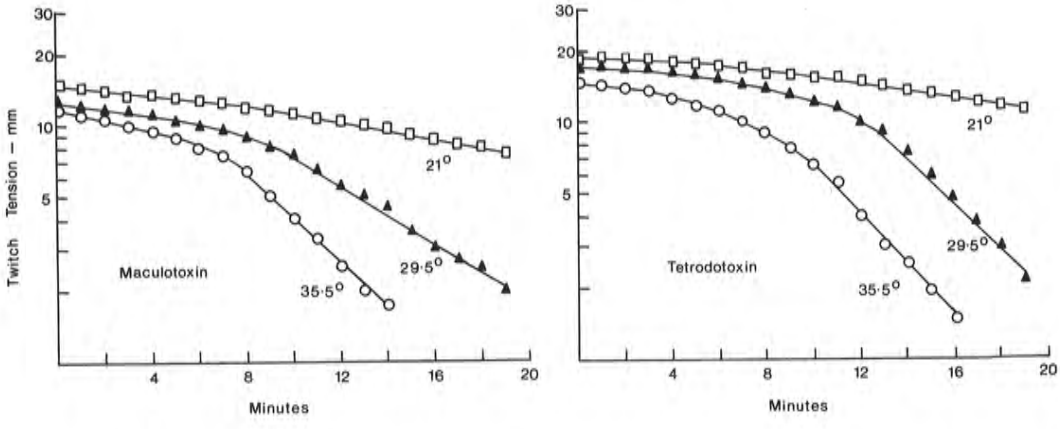


Figure 20: The onset of neuromuscular blockade in the isolated rat phrenic nerve-diaphragm preparation after equivalent doses of maculotoxin and tetrodotoxin at 21°C - 35.5°C. Semilogarithmic coordinates; 20mm twitch tension = 18.2g.

to avoid residual effects of retained toxin. Because of this effect minimum doses consistent with complete blockade were always used. The rate of development of blockade showed the same steep dose dependency associated with lethality in the intact animal.

Both maculotoxin and tetrodotoxin were more effective as neuromuscular blocking agents at high temperature than at low temperature. Figure 20 illustrates the effect of temperature on equivalent doses of MTX and TTX. Toxin doses that were almost without effect at 21°C caused complete blockade in 16 - 17 minutes at 35.5°C. In a further series of experiments it was found that equivalent doses of both toxins (calibrated as mouse units) caused neuromuscular blockade at a similar rate, when both latency and time to half decay of tension were compared.

Denervated diaphragm

Acetylcholine dose-response curves were plotted for the chronically denervated rat diaphragm (Freeman and Turner, 1969b) and were repeated after the addition of maculotoxin to the organ bath. Doses of MTX ranging from 0.3 - 1.0 M.U. per ml produced no direct effect on the diaphragm, neither was the acetylcholine dose-response curve altered by the presence of the toxin. The toad rectus abdominus preparation was similarly unaffected by maculotoxin. These two preparations are also insensitive to tetrodotoxin and saxitoxin (Freeman and Turner, 1969b; Kao, 1966).

Sciatic nerve preparation

As was found previously (Simon et al., 1964) maculotoxin caused a reversible blockade of the desheathed

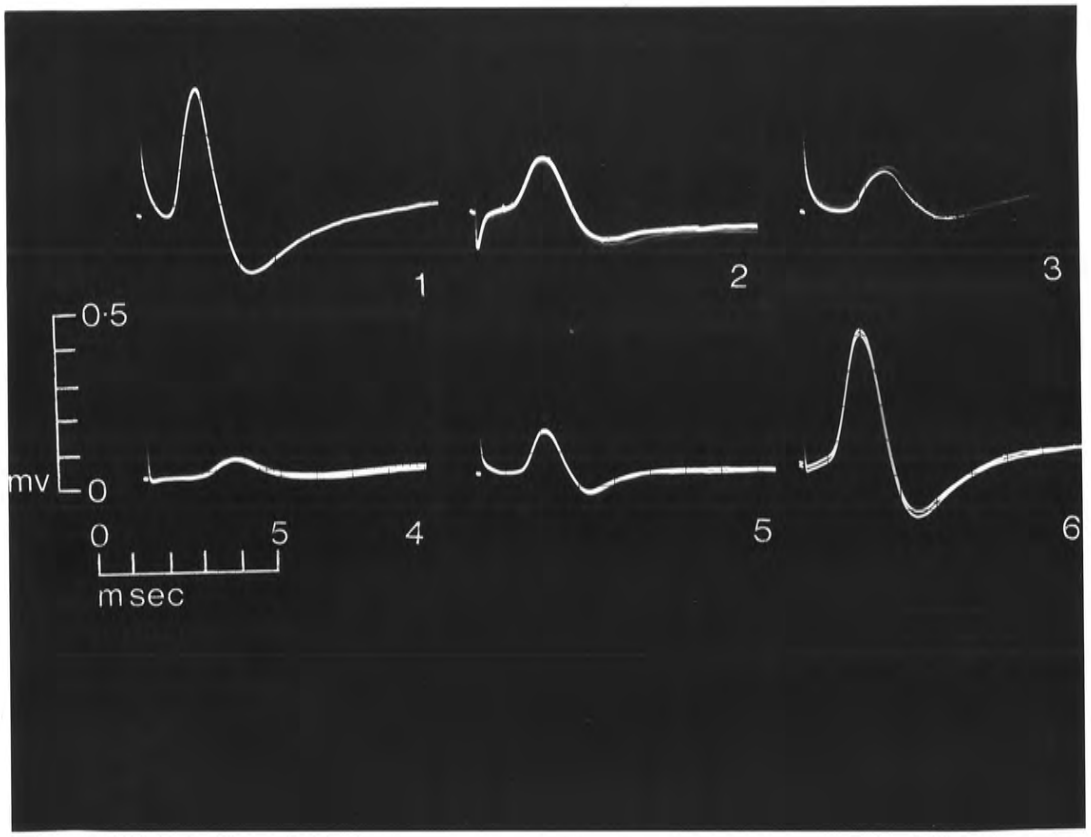


Figure 21: Effect of 0.02 M.U./ml maculotoxin on desheathed toad sciatic nerve. 1: Control action potential (AP). 2, 3, 4: AP 40, 60 and 90 minutes after MTX. At 90 minutes the period between the stimulus artefact and the peak of the AP had increased by 50%; the width of the AP had also increased by 50%. Washout of MTX was commenced immediately after 4. 5,6: 20 and 40 minute wash. 6 is identical with the control.

toad sciatic nerve. Doses of 0.02 M.U. per ml caused widening of the compound action potential and a decrease in conduction velocity prior to transmission block (see figure 21). Rat sciatic nerve was similarly blocked by maculotoxin. The rat sciatic nerve was difficult to desheath, so the relatively fine peroneal branch was used in these experiments.

Guinea pig ileum

Maculotoxin caused a slight contraction of the ileum preparation. This effect was blocked by diphenhydramine (10^{-7}g/ml) but was unaffected by atropine or lysergic acid diethylamide. It is possible that the gland extract contained some histamine which was not completely separated by Sephadex chromatography. The presence of 0.25 M.U. per ml maculotoxin did not affect the acetylcholine-induced contraction of the ileum over a wide concentration range.

Perfused guinea pig heart

Maculotoxin (10 M.U.) was injected into the perfusion system just proximal to the heart. This produced a marked reduction in coronary flow rate and a coincident increase in heart rate over the first 30 seconds, followed by a return of both parameters to control levels after one minute. Isotonic contraction amplitude increased over the first minute and then decreased to about 30 per cent of the control value for 2 - 3 minutes with a slow return to normal over the following 5 minutes. Doses less than 5 M.U. were without any observable effect on the heart.

The cardiac effects of maculotoxin are probably

insignificant in the overall toxin response since the amounts required to affect the heart are more than sufficient to cause rapid death through respiratory paralysis and circulatory insufficiency resulting from hypotension.

DISCUSSION

The pharmacology of the toxin of O. maculosus as described here is consistent with the earlier reports of Simon et al. (1964) and Trethewie (1965). In the present investigation, however, the results of chromatographic studies of salivary gland extract, using Sephadex G-25, suggest that the toxic effects are due to only one compound, whereas earlier papers by Simon et al. (1964) and Sutherland and Lane (1969) report that there are two chromatographically distinct toxins. The elucidation of this point must await further studies.

The pharmacological properties of maculotoxin resemble those of tetrodotoxin and saxitoxin in that death after envenomation is due to respiratory paralysis accompanied by profound hypotension. In addition, the molecular weight of MTX is similar to that of STX, as shown by Sephadex chromatography, and all three toxins behave identically on Amberlite IRC-50 cation exchange resin. Maculotoxin preparations which have been calibrated as 'mouse units' show quantitatively similar effects to tetrodotoxin when compared in a variety of pharmacological tests. The occurrence of hypotension at doses just sufficient to produce respiratory depression suggests that the toxin may be more closely related to tetrodotoxin than to saxitoxin (Kao and Nishiyama, 1965).

The failure of respiration after lethal doses of maculotoxin was characterized by a loss of diaphragmatic activity which preceded the failure of phrenic nerve volleys. For a short time these actually increased in

amplitude, no doubt due to the effect of hypoxia on the respiratory centre. However, the increase in phrenic activity was at no time comparable to that seen after the administration of a neuromuscular blocking agent such as gallamine or d-tubocurarine. The sequence of the effects which lead to respiratory failure may reflect the rate at which the toxin is carried to various tissues by the circulation. Thus the relatively greater vascularity of the diaphragm may result in blockade of the fine, intramuscular nerve axons before this is evident in the phrenic nerve trunk. Any central effects are also likely to be delayed by diffusion across the blood-brain barrier. Cheng et al. (1968) have discussed such effects in regard to respiratory failure after tetrodotoxin poisoning.

The profound hypotension observed after administration of maculotoxin is likely to result from generalized vasodilatation rather than a fall in cardiac output. Little or no change in pulse pressure is observed even after doses which reduce the arterial pressure by 50 per cent, andthe isolated heart preparation is similarly unaffected except at high doses of toxin. Vasodilatation may result from blockade of peripheral vasomotor nerves and/or the spinal vasomotor areas as proposed by Kao et al. (1967) for tetrodotoxin and saxitoxin.

The lack of effect of maculotoxin on either the nicotinic or muscarinic acetylcholine receptors, as shown by experiments with the denervated diaphragm, toad rectus abdominis and guinea pig ileum preparations, respectively, further demonstrates the similarity of maculotoxin to

tetrodotoxin and saxitoxin. Local anaesthetic drugs such as procaine, which block axonal transmission and cause hypotension, also have anticholinergic effects.

Resuscitation by artificial ventilation of an intoxicated animal, required that the animal have a marginally lethal dose, that ventilation be instituted before cyanosis became evident, and that it be prolonged for some time after the return of spontaneous respiratory movements. Although the anaesthetized animal cannot be compared with man, this finding agrees with the clinical observation that partial respiratory paralysis may last for up to eight hours or more.

At this time no specific antidote is available for the neurotoxins related to tetrodotoxin, although prolonged artificial respiration may lead to complete recovery in victims who have received marginally lethal doses of toxin. Attempts to produce immune responses to O. maculosus toxin in animals (Sutherland and Lane, 1969) have so far been unsuccessful.

STUDIES OF THE CHOLINERGIC RECEPTOR IN MAMMALIAN STRIATED MUSCLE

INTRODUCTION

The literature survey on cholinergic mechanisms is not intended to be as detailed as that on marine toxins. The mammalian neuromuscular junction has been the subject of extensive investigations and although a great amount has been learned about the structure of the junction and the processes involved in neuromuscular transmission, many details have yet to be explained. The aim of the present survey is to provide a background for the experiments to be described in this section and in the published papers 1, 2, 4 and 7 (see Appendix 4) and to provide a reference source to the many aspects of this topic.

STRUCTURE OF THE MOTOR END-PLATE

The fine structure of the neuromuscular junction has been the subject of investigations by many workers. The electron microscopy studies of Andersson-Cedergren (1959) may be consulted for a detailed description of the junction; studies by Reger (1959) and Couteaux (1958 and 1960) are also informative.

The mammalian motor end-plate is 20 - 30 microns in diameter and is composed of an axon terminal, an investing Schwann cell and a zone of modified sarcoplasm. The nerve axon divides into several fine branches which terminate in synaptic gutters, shallow depressions in

the muscle fibre surface. The terminal nerve branches are unmyelinated but are accompanied by Schwann cells (teloglia). At the nerve-muscle interface the sarcoplasmic membrane is deeply infolded to form the subneural apparatus, a complex structure which is separated from the underlying myofibrils by a thick layer of sarcoplasm. The axon membrane follows the general outline of the synaptic trough but does not penetrate the subneural folds. Nerve and muscle membranes are separated by the synaptic cleft, a 500A gap filled with an amorphous basement membrane material.

SEQUENCE OF EVENTS IN NEUROMUSCULAR TRANSMISSION

Although the conduction of impulses in nerve and muscle is electrical, measurements of synaptic delay (Katz and Miledi, 1965) have shown that there is a discontinuity in transmission at the neuromuscular junction. The early experiments of Dale, Feldberg and Vogt (1936) suggested that acetylcholine (ACh) may act as a chemical mediator and this has since been confirmed (for references see Katz, 1966; Karczmar, 1967).

Synthesis and release of acetylcholine

Motor nerve fibres have been shown to contain both ACh and the enzyme, choline acetylase (Nachmansohn, 1963) needed for its synthesis. ACh is thought to be stored in submicroscopic (400A) vesicles in the terminal region of the axon (Koelle, 1962). The presynaptic release of ACh is triggered by electrical impulses reaching the motor axon terminals. The process requires the presence

of calcium ions, possibly as components of a carrier mechanism which is involved in the release of quantal packets of transmitter (Katz and Miledi, 1968). Decrease of the normal external Ca^{++} concentration produces a decrease in the amount of ACh released by a nerve impulse. An increase in the external Mg^{++} concentration produces the same effect, possibly due to competition with Ca^{++} in the carrier system (del Castillo and Engbaek, 1954; Jenkinson, 1957; Karczmar, 1967).

Other factors including the storage and synthesis of ACh, mobilization of the transmitter and positive feedback actions of ACh upon the nerve terminal may also influence ACh release (Karczmar, 1967). Koelle (1962) suggests that the ACh released by the nerve action potential may act presynaptically to trigger release of greater amounts of ACh for neuromuscular transmission.

The postsynaptic process

After release from the nerve terminal, ACh diffuses across the synaptic cleft and unites with a stereospecific receptor located in the postsynaptic membrane. This reaction is accompanied by a large, non-specific increase in permeability to small cations. The subsequent membrane depolarization (end-plate potential) is produced by an inward flux of Na^{+} and is limited in size by a simultaneous outward movement of K^{+} (Katz, 1966). The end-plate potential is a graded response which is dependent on the amount of ACh released. If this is sufficient to cause a membrane depolarization of 50 - 60mV, a muscle action potential is produced. The action potential

results from a sequential rise in Na^+ conductance then K^+ conductance which causes a transient increase in potential to approximately +40mV, with a rapid return to the resting level.

The action of ACh at the muscle membrane is rapidly terminated by hydrolysis by the enzyme acetylcholinesterase (AChE), which has been shown by histochemical methods to be highly concentrated at the neuromuscular junction especially on the postsynaptic surface (Barrnett, 1962; Guth, 1968).

It is reasonable to assume that the catalytic site of AChE and the binding site of the physiological ACh receptor are likely to have some common features. Changeux, Podleski and Meunier (1969) discuss the possibility that AChE may possess regulatory sites, distinct from the active centre, which could play a physiological role as ACh receptor sites. This hypothesis is supported by the observation that AChE is able to bind ACh even when the esteratic site is blocked by eserine.

These workers emphasize that such an hypothesis does not exclude the possibility that proteins other than the AChE subunits may contribute to the ACh macromolecular receptors, and they further suggest that there may be several classes of ACh receptor sites of which the regulatory sites of AChE may be one.

For muscle contraction to occur, the effect of membrane excitation must be conducted inwards from the surface membrane of the muscle cell to the actin-myosin contractile system in the centre. This excitation-contraction coupling is achieved through the sarcotubular

system, an intracellular network of fine tubules which forms an electrical extension of the surface membrane, thus reducing the distance between the points of electrical and mechanochemical response (Franzini - Armstrong and Porter, 1964; Huxley and Taylor, 1958; Ebashi, 1965).

Ca^{++} release from this system into the sarcoplasm and subsequent reactions involving the hydrolysis of ATP ultimately lead to an interaction between myosin and actin filaments producing contraction (for references see Huxley, 1964).

Alterations in the external concentrations of divalent cations may modify the postsynaptic processes at several stages. The size of the end-plate potential is dependent on the external concentrations of both Ca^{++} and Mg^{++}. Increases in either of these cations have a stabilizing effect on the postsynaptic membrane, thus reducing the sensitivity of the end-plate to ACh. As mentioned above, Ca^{++} is also involved in the excitation-contraction coupling mechanism. Membrane depolarization does not produce a mechanical response in skeletal muscle in the absence of Ca^{++} (Frank, 1964); high external levels of Mg^{++} antagonize the action of Ca^{++} in excitation-contraction coupling.

EFFECTS OF DENERVATION ON MAMMALIAN SKELETAL MUSCLE

The chronic section of a motor nerve leads to several structural and functional changes in the skeletal muscle.

Structural changes

Detailed investigations of the morphological effects of denervation on the mammalian neuromuscular junction and skeletal muscle have been made by Miledi and Slater (1968, 1970) and Pellegrino and Franzini (1963).

In the rat phrenic nerve-diaphragm preparation, transmission failure occurs abruptly at all end-plates. Failure is accompanied by a cessation of spontaneous miniature end-plate potentials and, unlike denervated amphibian muscle, there is no significant resumption of this activity at a later stage.

For 8 - 12 hours after section of the nerve, nearly all end-plates appear normal. This latent period is followed by a phase during which all the end-plates in the muscle undergo a process of breakdown; after 18 - 20 hours all appear grossly abnormal and nearly all have ceased to function.

In the first few days after denervation the axon terminal undergoes a process of fragmentation and the Schwann cell cytoplasm fills the synaptic groove, effectively replacing the axon. The Schwann cell withdraws over the next 3 - 4 weeks and the space previously occupied by the axon and the Schwann cell is traversed by collagen fibrils. Synaptic gutters tend to disappear after axon disintegration, but the synaptic folds of the subneural apparatus remain for at least five months.

Changes in sensitivity to acetylcholine

ACh produces membrane depolarization in innervated

muscle only when applied to the end-plate region (less than 0.1mm of fibre length) (Axelsson and Thesleff, 1959). After denervation, however, the acetylcholine sensitive area of the mammalian muscle fibre membrane spreads from the end-plate region until at 6 - 8 days it uniformly covers the entire fibre surface (Axelsson and Thesleff, 1959). This allows ACh to produce a graded depolarization over the entire length of the muscle fibre and so initiate a contraction unaccompanied by conducted responses (Elmqvist and Thesleff, 1960). Whereas the propagation of an action potential along a muscle fibre causes a transient contraction (twitch), the response to the persistent depolarization produced by ACh in denervated muscle is a more prolonged increase in tension.

The reasons for the spread of ACh sensitivity in denervated muscle are not understood. Studies by Thesleff (1960) have shown that treatment of mammalian skeletal nerve-muscle areas with botulinum toxin, which prevents presynaptic release of ACh without causing any observable structural changes, produces similar effects to those observed after chronic denervation. There is also evidence that factors other than the prevention of ACh release may be involved (for references see Guth, 1968). The ability to produce ACh-receptive sites may be inherent in all areas of the muscle membrane; possibly some neural influence normally restricts this to the immediate surroundings of the neuromuscular junction (Katz and Miledi, 1964).

Effects of denervation on the electrical properties of skeletal muscle

The resting membrane potential of the skeletal muscle cell is decreased by denervation; experiments with the rat diaphragm muscle have shown that the membrane potential changes from 74 ± 1mV (± S.E. of 69 observations) to 67.8 ± 0.7mV (± S.E. of 37 observations) (Freeman and Turner, unpublished).

ACh produces a graded depolarization in denervated muscle which persists for some time after the muscle has relaxed. The ACh-induced contracture of the denervated rat diaphragm has a time course of less than 90 seconds (Freeman and Turner, 1969b); constant levels of membrane depolarization have been observed for 8 - 10 minutes (Freeman and Turner, unpublished). The reason for the brevity of the ACh-induced contraction has yet to be explained, but the mechanism may involve the resequestration of Ca^{++}, released by the depolarization, by the sarcoplasmic reticulum even though the membrane remains depolarized.

After denervation, the muscle fibres remain electrically excitable and a sufficiently large depolarizing current will trigger a propagated action potential. The threshold membrane potential at which excitation occurs is not altered by denervation (Axelsson and Thesleff, 1959).

Following degeneration of the motor nerve spontaneous action potentials may be detected in a small percentage of denervated muscle fibres (Thesleff, 1963; Freeman and Turner, unpublished). These spikes are characterized by

a regular frequency and always start from slowly rising prepotentials. Thesleff (1963) suggests that one of the causes of fibrillation potentials is an alteration in the potassium conductance of the muscle cell membrane. The decrease in membrane potential is also likely to contribute to fibrillation.

Effect of denervation on acetylcholinesterase levels

For the first 10 days after denervation the level of end-plate AChE shows a marked decrease. Thereafter, a slow decrease in AChE activity is observed, although weak histochemical reactions can be detected up to eight months after denervation (Eränkö and Teräväinen, 1967).

Effects of drugs on denervated muscle

The response of the chronically denervated muscle to many drugs is qualitatively similar to that of the innervated end-plate (Axelsson and Thesleff, 1959).

Acetylcholine, carbachol and tetramethylammonium (TMA) produce dose-dependent depolarizations and contractions in denervated muscle. The studies of Elmqvist and Thesleff (1960) and Freeman and Turner (1969b) have shown that d-tubocurarine (d-TC) competitively inhibits the response of the denervated diaphragm muscle to ACh, carbachol and TMA, in concentrations which cause partial or complete neuromuscular blockade of the innervated muscle. Beránek and Vyskočil (1967), however, reported that denervated muscle is less sensitive to d-TC, and Loomis and Konker (1967) found that d-TC produced a contracture of the denervated anterior tibial muscle of the rat.

Effect of alteration of the external ionic environment

Experiments by Freeman and Turner (1969b) have shown that reduction of external Ca^{++} or Mg^{++} concentration to one-tenth normal had no significant effect on the ACh-induced contraction in the denervated rat diaphragm. Increases in the level of either cation, however, reduced the magnitude of the ACh contraction, and in the case of Ca^{++}, greatly increased the time to half relaxation.

The decreased tension developed in high Ca^{++} or Mg^{++} solutions may be due to the membrane stabilizing effects of these ions; the prolongation of the contraction by increased Ca^{++} may be a function of the ability of the sarcoplasmic reticulum to resequester Ca^{++} under conditions of continuing depolarization and an increased Ca^{++} gradient.

EFFECTS OF FACILITATORY DRUGS AT THE NEUROMUSCULAR JUNCTION

The experiments to be described in this section of the thesis concern the actions of facilitatory drugs at the neuromuscular junction.

Drug-induced facilitation of the indirectly elicited muscle twitch may, in theory, result from an action of the drug at a number of points. The facilitatory drug may act presynaptically by effectively increasing the amount of ACh released by the nerve impulse (Werner, 1960; Standaert and Riker, 1967; Riker and Okamoto, 1969). Alternatively, the drug could act postsynaptically by end-plate sensitization or by an effect on the process of

excitation-contraction coupling (Katz and Thesleff, 1957; Karczmar, Kim and Blaber, 1965; Karczmar, 1967). The anticholinesterase activity of many of the facilitatory drugs may also be a contributing factor.

The occurrence of drug-induced facilitation in the isolated phrenic nerve-diaphragm preparation of the rat has enabled a study of facilitatory drug action and the effects of alteration of temperature and external ionic environment on the facilitatory process to be made. In this way some indication of the relative importance of pre- and post-synaptic factors has been obtained.

The effect of facilitatory drugs on the ACh receptors of the chronically denervated diaphragm of the rat has also been studied. As previously stated, the receptors induced in the diaphragm by denervation show some quantitative differences from the innervated post-synaptic receptor. However, the preparation has been shown to offer a reasonable method for examining ACh-receptor interaction and it has certain advantages over the intact junction. The receptor is easy to approach experimentally; drug concentrations and ionic levels can be maintained or varied as required. The preparation contains cholinesterase only in the region of the degenerating synapse (Eränkö and Teräväinen, 1967).

The effects of four facilitatory drugs have been determined on innervated and denervated diaphragm preparations. Interactions with succinylcholine (SCh) have also been studied, to determine the role of facilitatory processes in the relief of SCh blockade.

RESULTS

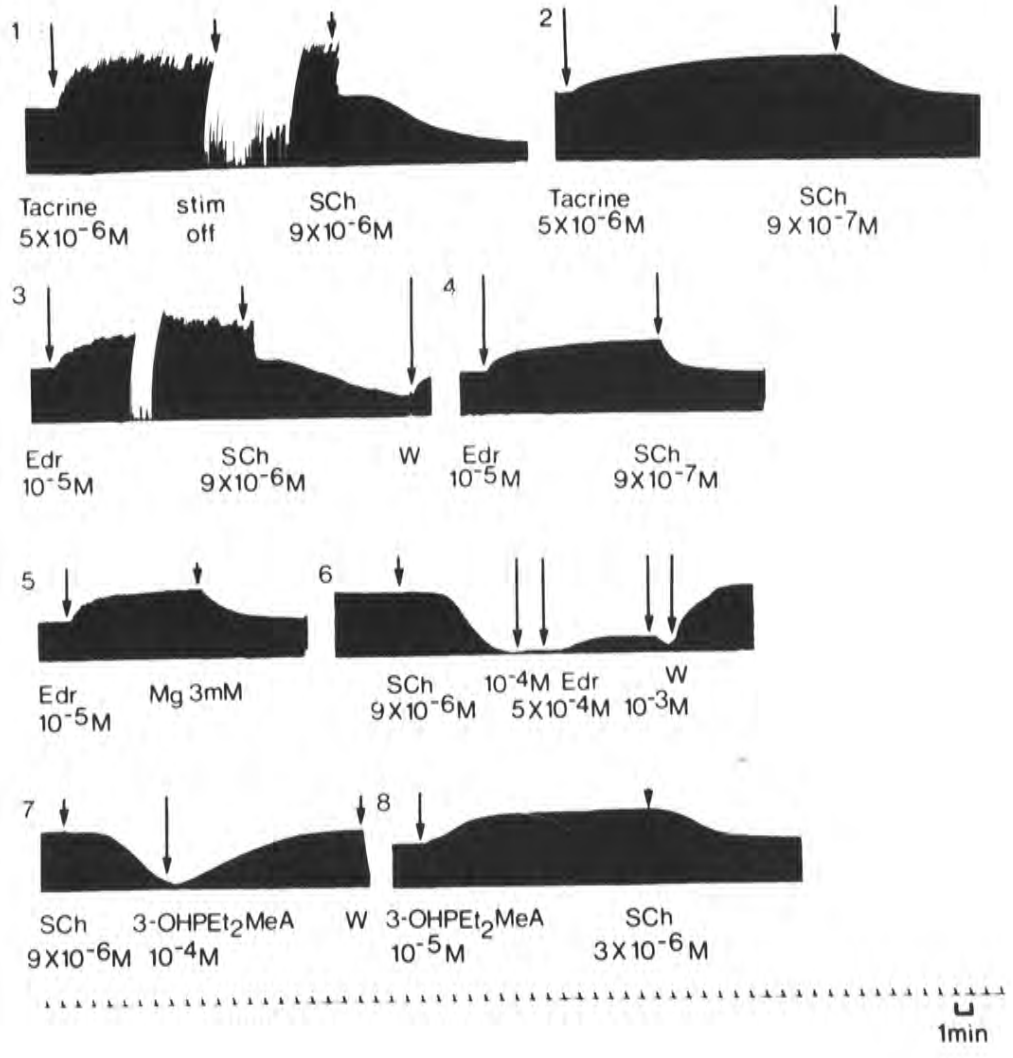
The facilitatory drugs used were tetrahydro-4-aminoacridine hydrochloride (tacrine), 3-hydroxyphenyldimethylethylammonium chloride (edrophonium), 3-hydroxyphenyldiethylmethylammonium bromide (3-OHPEt$_2$MeA) and 3-hydroxyphenyltriethylammonium bromide (3-OHPTEA).

Effects of facilitatory drugs on neuromuscular transmission

All of the compounds studied produced facilitation of the isometric twitch. Contraction amplitude increased over a period of 5 - 8 minutes after addition of the drug to the organ bath, and the increase persisted for the time of exposure of the preparation to the drug. In the case of the three hydroxyanilinium compounds the effect subsided when the preparation was washed, however, potentiation due to tacrine persisted for more than 40 minutes after washout of doses less than 5×10^{-6}M, and this effect was virtually irreversible at higher doses. All compounds except 3-OHPTEA caused some degree of spontaneous twitching; such twitching was particularly marked with tacrine (see figure 22, panel 1), although it varied among preparations.

As has been reported in experiments *in vivo* (Kuperman, Gill and Riker, 1961; Blaber and Karczmar, 1967b), the drug concentration for optimal facilitation showed a large variation. In the present investigation the concentration range extended from 5×10^{-6}M for tacrine to 10^{-3}M for 3-OHPTEA. Figure 23 shows dose-response curves for tacrine, edrophonium and 3-OHPEt$_2$MeA.

Figure 22: Effects of facilitatory drugs on phrenic nerve-diaphragm preparations; interactions between these drugs and succinylcholine (SCh).
1: tacrine (5×10^{-6}M) added at first arrow; stimulator switched off for 4 minutes at second arrow. Twitch potentiation and spontaneous activity suppressed by SCh (9×10^{-6}M) added at third arrow.
2: tacrine produced twitch potentiation but less spontaneous activity. SCh (9×10^{-7}M) suppressed potentiation without causing blockade.
3: edrophonium (10^{-5}M) added at arrow. Spontaneous activity and twitch potentiation suppressed by SCh (9×10^{-6}M).
4: twitch potentiation due to edrophonium blocked by SCh (9×10^{-7}M) without subsequent blockade.
5: potentiation due to edrophonium was abolished by 3mM Mg^{++}.
6: SCh blockade followed by the sequential addition of edrophonium (10^{-4}M, 5×10^{-4}M, 10^{-3}M); only 5×10^{-4}M significantly relieved blockade.
7: SCh blockade relieved by 10^{-4}M 3-OHPEt$_2$MeA.
8: potentiation due to 3-OHPEt$_2$MeA (10^{-5}M) suppressed by SCh (3×10^{-6}M). Twitch tension in the absence of drugs 10 - 13g; time marker = 1 minute.


1
Tacrine
5X10-6M
stim
off
SCh
9X10-6M
2
Tacrine
5X10-6M
SCh
9X10-7M
3
Edr
10-5M
SCh
9X10-6M
W
4
Edr
10-5M
SCh
9X10-7M
5
Edr
10-5M
Mg 3mM
6
SCh
9X10-6M
10-4M
Edr
5X10-4M
W
10-3M
7
SCh
9X10-6M
3-OHPEt2MeA
10-4M
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10-5M
SCh
3X10-6M
1min

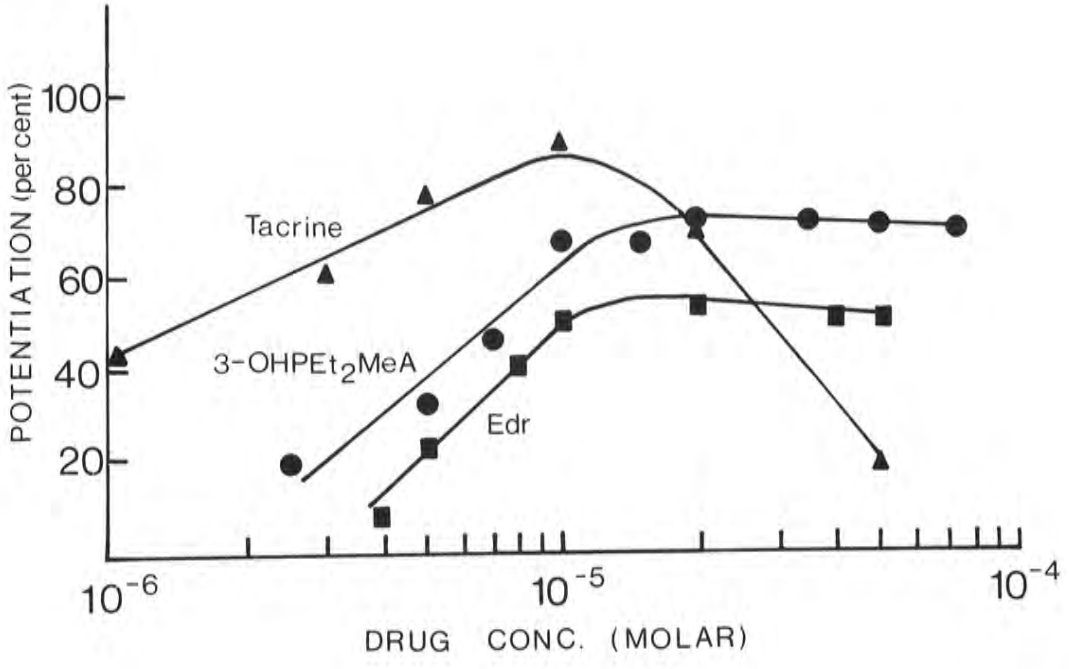


Figure 23: The relationship between percent increase in twitch tension and drug concentration for tacrine (▲ - ▲), 3-OHPEt$_2$MeA (● - ●) and edrophonium (■ - ■). Each point is the mean of 6 - 9 observations. Stimulation rate = 9/minute. Semi-logarithmic scale.

The slopes of the linear portions of the dose-response curves were not significantly different at the 5% probability level. The curve for 3-OHPTEA resembled that of the other two hydroxyanilinium compounds except that a hundredfold greater drug concentration was required. Tacrine produced twitch depression at dose levels closer to those required for maximal twitch potentiation than did the hydroxyanilinium compounds.

The twitch potentiation which followed addition of each of the drugs was frequency dependent. It was greatest at stimulation rates between 6 and 12 per minute, but decreased to zero at rates greater than one per second. In all instances, the frequency dependence was tested using drug concentrations which produced optimal potentiation.

Effect of temperature on facilitation

The facilitatory process was found to be sensitive to the temperature of the bathing solution. Potentiation and spontaneous firing, which were maximal at 36°C, were abolished when the temperature was lowered to 20°C. Experiments were therefore carried out to estimate the apparent energy of activation (E_a) of the facilitatory process. Since the normal process of neuromuscular transmission is itself temperature sensitive, the experiments were designed so that twitch tension was determined at each temperature before the addition of the drug to the organ bath. In the case of tacrine, where the drug could only be washed out of the preparation very slowly, the twitch tension was determined over the

temperature range 20° - 36°C before drug addition. The temperature was then varied over the same range in the presence of the drug, and the percentage twitch potentiation was determined. The concentration of tacrine was 5×10^{-6}M, and that of edrophonium was 10^{-5}M.

Results were plotted as the logarithm of the percentage increase in tension against the reciprocal of the absolute temperature, and the apparent energy of activation, E_a, was derived from the Arrhenius equation using the gradient of the statistically determined regression.

Several assumptions are implicit in making such calculations: firstly, that potentiation is proportional to the rate of reaction, secondly, that one reaction in the sequence leading to potentiation is dominant and provides a rate-determining step, and thirdly, that this reaction retains its dominance over the temperature range studied. Some support for the latter two assumptions is given by the fact that an analysis of variance showed that there was a highly significant linear correlation between $\log_{10}$ potentiation and the reciprocal of the absolute temperature.

For tacrine, $E_a = 25.2 \pm 1.2$ kcal mol^{-1} (95% confidence limits); for edrophonium, $E_a = 26.7 \pm 3.3$ kcal mol^{-1} (95% confidence limits). The figures are not significantly different at the 5% probability level. It is of interest to note the high values of E_a obtained, and to compare them with the value of approximately 48 kcal mol^{-1} obtained by Hofman, Parsons and Feigen (1966) for E_a of the miniature endplate potential frequency in the rat diaphragm.

Effect of changes in external divalent cation levels

Twitch potentiation was sensitive to small changes in the divalent cation level of the bathing solution. Increasing the external Ca^{++} concentration to 3.0 or 4.5mM (normal level 1.5mM) increased the drug-induced potentiation produced by all the hydroxyanilinium compounds but had no effect on the response to tacrine. It was noted that 3.0mM Ca^{++} increased twitch potentiation by the hydroxyanilinium compounds more at low drug levels than at optimal levels. That is, the dose-response curve in high Ca^{++} diverged from the control curve, and the drug concentration necessary for optimal potentiation was reduced.

Reduction of the Ca^{++} concentration to 0.38mM (x0.25 normal) completely abolished twitch potentiation in all instances. If the Ca^{++} level was reduced to 0.15mM (x0.1 normal) a slow blockade of neuromuscular transmission occurred (Freeman, 1968b). Addition of drugs to the organ bath during the course of the low Ca^{++} blockade caused varying effects. Tacrine (5×10^{-6}M) produced a transient relief of blockade lasting for 1 - 2 minutes after which twitch tension continued to decrease at the original rate. This effect was also observed with edrophonium and 3-OHPEt$_2$MeA at concentrations up to 10^{-4}M. Higher drug concentrations increased the rate of onset of low Ca^{++} blockade. 3-OHPTEA (10^{-4}M) either brought about a slight relief of low Ca^{++} blockade or halted its progress. In this regard this compound resembles the structurally related drug triethylcholine (Freeman, 1968b).

Reduction of the external Mg^{++} concentration to

0.1mM (x0.1 normal) had no effect on twitch potentiation. However, small increases in external Mg^{++}, which themselves had no effect on twitch tension, completely abolished twitch potentiation. An increase in the external Mg^{++} concentration from the usual level of 1mM to 2mM was sufficient in all instances to bring about a slow reduction in twitch potentiation to the control level. 3 - 4mM external Mg^{++} reduced twitch tension to the control level in 3 - 4 minutes (figure 22, panel 5).

Thus ionic conditions that do not reduce transmitter release sufficiently to interfere with transmission can nevertheless abolish twitch potentiation.

Drug effects on the ACh receptor of the denervated diaphragm

The interactions of Ca^{++} and Mg^{++} with the facilitatory drugs in the innervated diaphragm suggest a presynaptic site for the process, and are consistent with the concept of Werner (1960) that these drugs produce positive and negative after potentials in the motor nerve terminals in response to nerve stimulation, thus modifying the transmission process. Other workers have, however, emphasized the importance of end-plate sensitization (Katz and Thesleff, 1957; Karczmar, Kim and Blaber, 1965).

The ACh receptors of the denervated diaphragm offer a convenient approach to the examination of ACh-receptor interaction. The preparation is easy to study experimentally and has the further advantage that it is largely free of acetylcholinesterase. This means that changes in receptor sensitivity cannot be attributed solely to anticholinesterase properties of the drugs.

Acetylcholine produced a dose-dependent increase in isometric tension in the denervated diaphragm. The time course of the contraction was relatively independent of the ACh concentration. The rise time increased slightly over the range 2.75×10^{-6}M - 2.75×10^{-5}M, but the time to half relaxation decreased from 64 seconds at 2.75×10^{-6}M to 31 seconds at 2.75×10^{-5}M.

After control dose-response curves for the ACh-induced contraction were obtained, the facilitatory drug was added to the organ bath and the ACh dose-response curve determined in the presence of the drug. The preparation was then washed for more than 30 minutes to remove the facilitatory drug, and the ACh contraction was rechecked to ensure that the sensitivity of the preparation had not altered. It was not always possible to carry out this final stage when tacrine was used, because of the slowness with which this drug was washed from the preparation. At least three levels of each drug were used.

Typical dose-response curves in the presence and absence of the facilitatory drugs are shown in figures 24 and 25. Data were not pooled for these figures as the slope of the ACh dose-response curve tended to vary slightly among preparations (Freeman and Turner, 1969b).

Tacrine, edrophonium and 3-OHPEt_2MeA all potentiated the ACh-induced contraction at concentrations which potentiated twitch tension in the intact phrenic nerve-diaphragm. The data for the potentiated ACh dose-response curves show more scatter than those of the control curves,

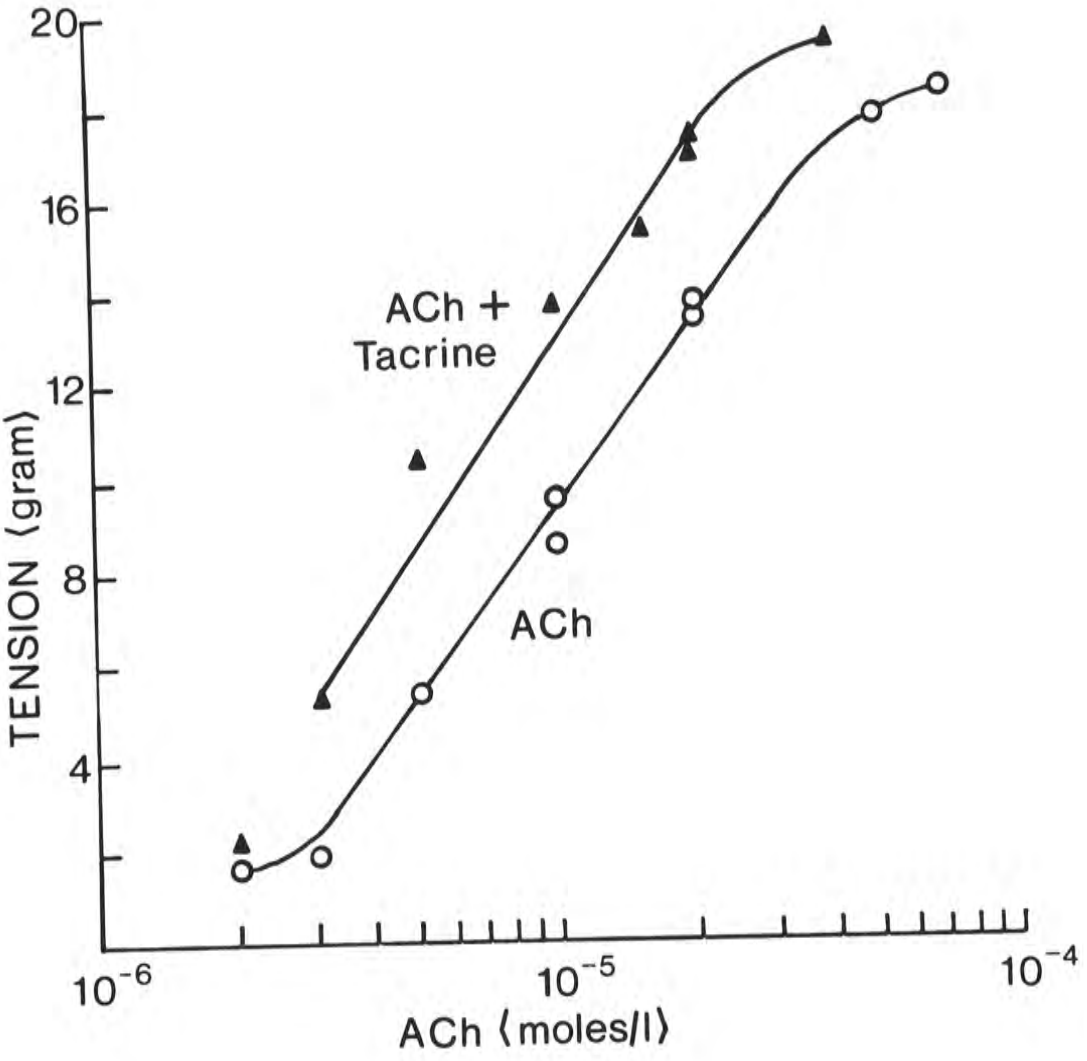


Figure 24: Potentiation of the ACh-induced contraction of denervated rat diaphragm by tacrine (5×10^{-6}M). ACh (O - O), ACh + tacrine (▲-▲). Semilogarithmic scale. Each point refers to one observation. ACh response after wash-out of tacrine was routinely checked at 10^{-5}M ACh.

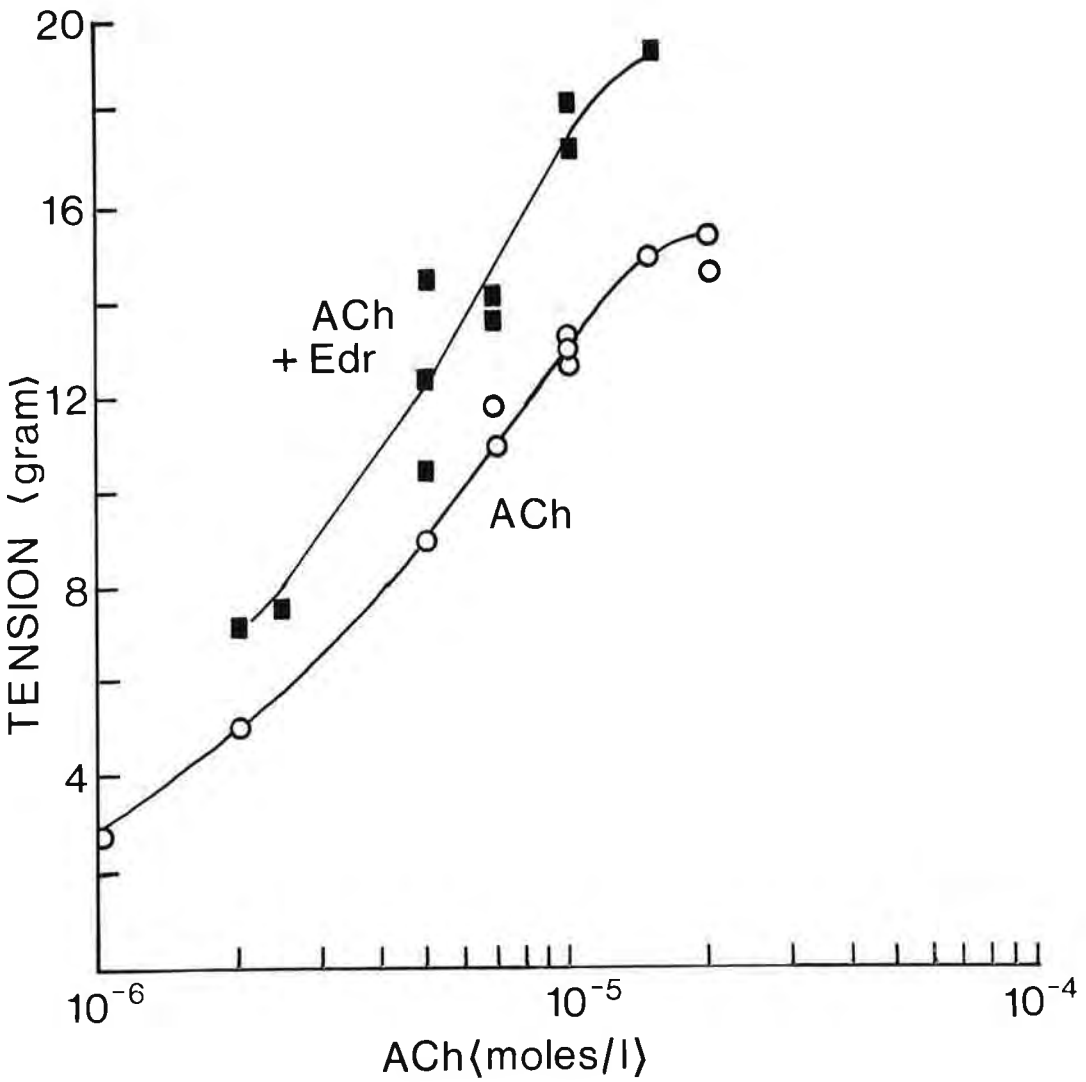


Figure 25: Potentiation of the ACh-induced contraction of denervated rat diaphragm by edrophonium (5×10^{-6}M). ACh (o - o), ACh + edrophonium (■ - ■). Semilogarithmic scale. Each point refers to one observation. ACh response after edrophonium wash-out was routinely checked at 10^{-5}M ACh.

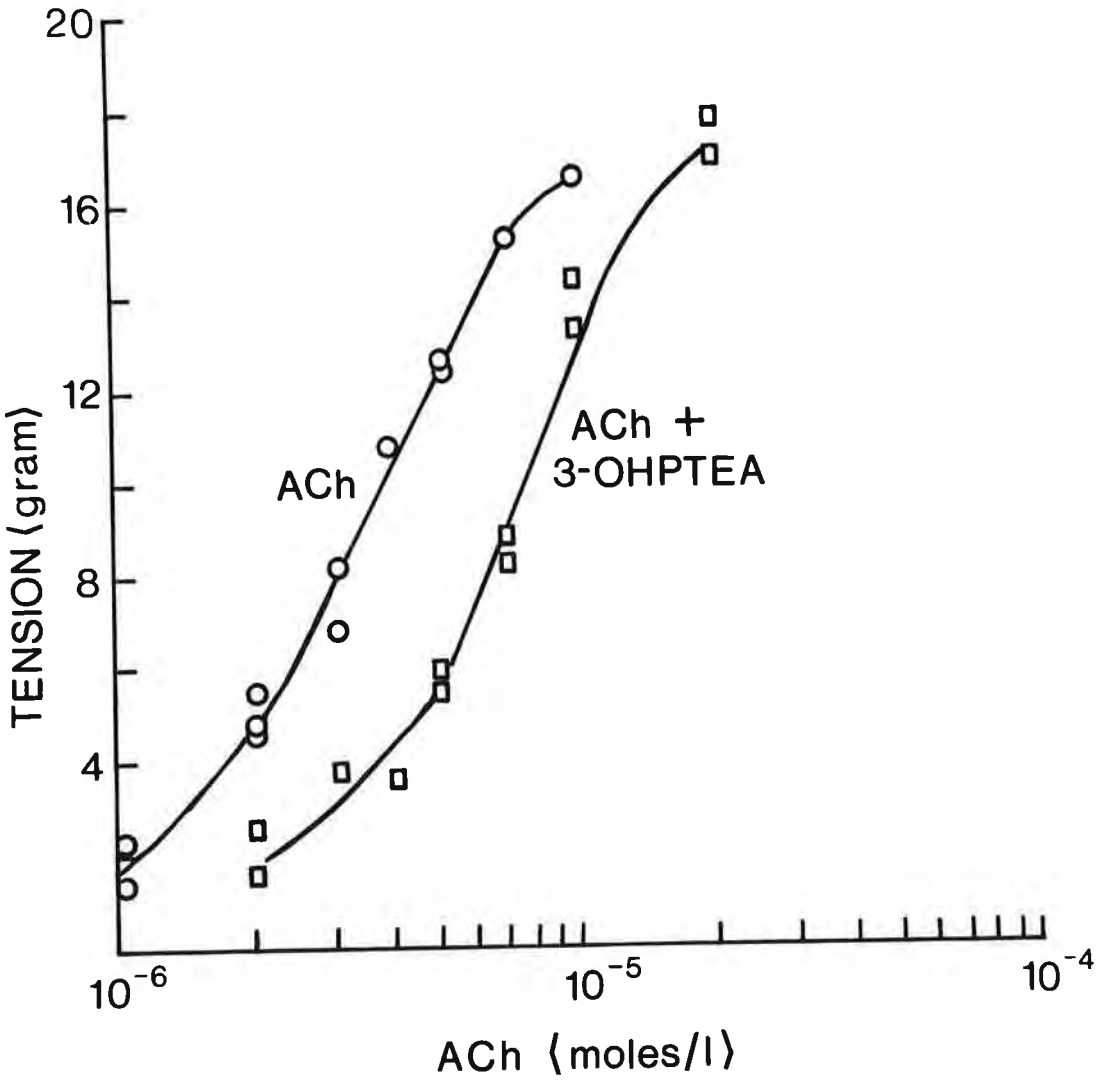


Figure 26: Depression of the ACh-induced contraction of denervated rat diaphragm by 3-OHPTEA (10^{-4}M). ACh (O - O), ACh + 3-OHPTEA (□ - □). Semilogarithmic scale. Each point refers to one observation. ACh response after wash-out of 3-OHPTEA was routinely checked at 10^{-5}M ACh.

and potentiation was consistently less at low levels of ACh than it was at levels producing nearly maximal contractions.

Greater concentrations (10^{-4}M) of edrophonium and 3-OHPEt$_2$MeA caused an initial potentiation of the ACh contraction which diminished with time of exposure to the drug until after approximately one hour, when these drug levels had a depressant effect. Similar drug levels also brought about twitch potentiation followed by depression in the intact phrenic nerve-diaphragm preparation.

In contrast to the findings with the other facilitatory drugs, 3-OHPTEA caused a depression of the ACh-induced contraction at levels which produced facilitatory responses in the innervated diaphragm (figure 26). Depression of the ACh contraction at 10^{-3}M 3-OHPTEA was so intense that doses of ACh greater than 10^{-4}M were needed to elicit a contraction. Dose-response curves at 10^{-4}M and 5×10^{-5}M 3-OHPTEA were parallel with the control, and the depression of the contraction could be entirely reversed by washing the preparation.

The effect of the facilitatory drugs on the depolarization of denervated muscle by ACh was also determined, since potentiation of the contraction could have been due to an effect on the process of excitation-contraction coupling rather than an increase in membrane depolarization.

The relationship between ACh concentration (10^{-6}M - 10^{-4}M) and depolarization (figure 27) was found

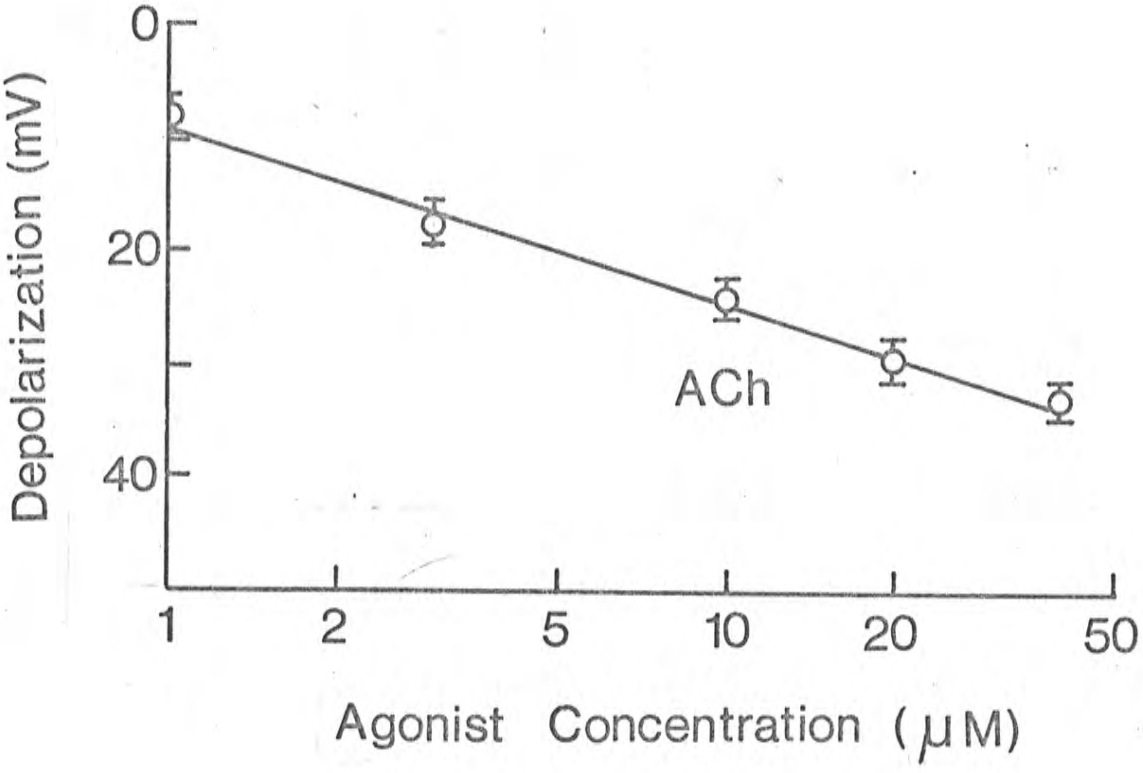


Figure 27: Depolarization of denervated diaphragm by ACh. Pooled data from diaphragms denervated for 8 - 13 days. Each point is mean ± S.E. of 21 - 37 readings. The control resting potential (68.3 ± 0.7, S.E. of 19 sets of readings) has been taken as zero depolarization. Semilogarithmic plot.

to conform closely to that reported by Lüllmann and Reis (1967). Experiments were designed so that the membrane potentials of a small population of muscle cells were determined before addition of ACh to the organ bath. A series of 6 - 8 penetrations were then made over a 4 minute period in the same area in the presence of ACh. The ACh depolarization of the denervated diaphragm was measured after the contraction had passed, since the contraction displaced the microelectrode from the muscle cell. Preliminary experiments, which confirmed the observations of Lüllmann and Reis (1967), had shown that the ACh depolarization remained constant for several minutes. The preparation was then washed for 20 minutes and a second group of readings taken and pooled with the first control group to obtain the ACh depolarization.

The effect of the facilitatory drugs on the ACh depolarization was determined at a fixed ACh concentration of 10^{-5}M. Groups of membrane potential measurements were made as before, and the effect of the facilitatory drug was estimated by subtraction of the depolarization due to ACh in the presence of the drug from the control membrane potential measured in the presence of the facilitatory drug alone. Optimal concentrations of the facilitatory drugs, as determined in the tension recording experiments, were used; these had no depolarizing effect on the muscle membrane.

The results of these experiments are shown in Table 8; the drugs potentiated the ACh depolarization at the same concentration as they potentiated the ACh-induced contraction.

Table 8. Effects of facilitatory drugs on the ACh depolarization of denervated muscle.

	ACh depolarization (mV)		
	Control	Treated	p
Edrophonium 10^{-5}M	26.4±1.9 (22)	35.9±1.3 (22)	0.001
3-OHPEt$_2$MeA 10^{-5}M	23.3±1.7 (15)	30.7±1.4 (15)	0.002
3-OHPTEA 10^{-4}M	29.0±1.6 (14)	14.8±1.7 (15)	0.001
Tacrine $5x10^{-6}$M	23.9±1.8 (17)	33.8±2.0 (15)	0.001

Figures shown are ± S.E.M. The number of observations is shown in parentheses. Significance of difference of means was determined using Student's t test. ACh concentration was 10^{-5}M. The control value for membrane potential was 67.8 ± 0.7mV (± S.E. of 37 sets of readings). This was not altered by these concentrations of the facilitatory drugs.

The ACh depolarization was depressed by 3-OHPTEA (10^{-4}M), as would be expected from the preceding data. This concentration of 3-OHPTEA did not depolarize the membrane; 10^{-4}M 3-OHPEt$_2$MeA also had no effect. However,

edrophonium (10^{-4}M) depolarized the membrane by 22mV, and tacrine (5×10^{-5}M) caused a barely significant depolarization of 5mV. These concentrations of edrophonium and tacrine caused a transitory potentiation of the indirectly-elicited muscle twitch, followed by depression.

It was noted that repeated exposure to ACh in the presence of a facilitatory drug over a period of several hours lessened the increase in depolarization. It is possible that repeated depolarizations of the membrane for 4 - 5 minute periods may have led to a reduction in ionic gradients which were not adequately reconstituted *in vitro*.

Sensitivity of the ACh-induced contraction to divalent cation variation

Previous experiments (Freeman and Turner, 1969b) had shown that the ACh-induced contraction of the denervated diaphragm was insensitive to reduction in the external concentration of Ca^{++} or Mg^{++} to 0.15mM or 0.1mM respectively. However, a threefold increase in the concentration of these ions (4.5mM or 3.0mM respectively) reduced the force of contraction, which was also prolonged by the high Ca^{++} concentration.

The sensitivity of the potentiated ACh contraction to divalent cation variation was determined for all three potentiating drugs to obtain a comparison with the ionic effects on drug action in the intact phrenic nerve-diaphragm preparation. The results of these experiments are summarized in figure 28. As noted previously, low Ca^{++}

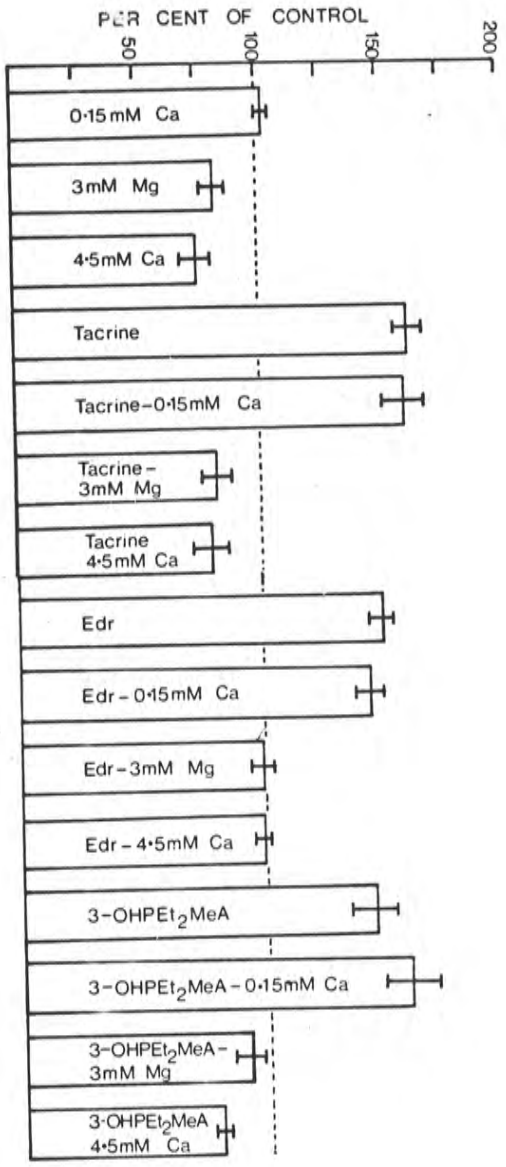


Figure 28: The effect of variation in divalent cation level on control ACh contraction of denervated diaphragm and ACh contraction in the presence of tacrine (5×10^{-6}M), edrophonium (5×10^{-6}M) and 3-OHPEt$_2$MeA (5×10^{-6}M). The dotted line indicates the control contraction to 10^{-5}M ACh in normal bathing solution. Error bars indicate $\pm$ S.E. of 6 - 7 observations.

had little or no effect, and high Ca^{++} or Mg^{++} depressed the ACh contraction. Potentiation of the ACh (10^{-5}M) contraction by tacrine (5×10^{-6}M) was insensitive to reduction in the external Ca^{++} or Mg^{++} concentration. Increased levels of these ions, however, reduced the tacrine-potentiated contraction to less than the control. Similar effects were observed when tacrine was replaced by edrophonium or 3-OHPEt$_2$MeA (figure 28).

It is of interest that the ionic effects on the drug-potentiated twitch of the intact phrenic nerve-diaphragm preparation are essentially opposite to those observed for the denervated diaphragm receptor. Low Ca^{++} concentrations abolish twitch potentiation, but have no effect on the potentiated ACh contraction. High concentrations of Ca^{++} or Mg^{++} have opposing effects on the intact junction, but both abolish potentiation in the denervated preparation.

Interactions between succinylcholine and the facilitatory drugs

Studies by Freeman (1968a, b) and Freeman and Turner (1968) have emphasized the importance of presynaptic factors in succinylcholine (SCh) blockade of the rat neuromuscular junction. Drugs which reverse SCh blockade were shown to act primarily by increasing the presynaptic release of ACh. Since the experiments reported in this study suggest that presynaptic factors are dominant in twitch potentiation of the intact diaphragm it was of interest to determine the ability of the facilitatory drugs to relieve SCh blockade.

Tacrine had no antidotal action against SCh blockade,

although the earlier observation of Ho and Freeman (1965), that prior treatment with tacrine reduced the intensity of blockade, was confirmed. The three hydroxyanilinium compounds all showed some antidotal action under these experimental conditions, although complete relief of SCh blockade was achieved only with 3-OHPEt$_2$MeA (figure 22, panel 7). 3-OHPTEA was less effective than the dimethyl derivative and edrophonium brought about an average restoration of twitch tension of only 33% (see Table 9).

Table 9. Percentage relief of SCh blockade by hydroxyanilinium compounds.

Concentration (M)	Edrophonium (%)	3-OHPEt$_2$MeA (%)	3-OHPTEA (%)
5×10^{-5}	-	93 ± 2 (5)	-
10^{-4}	no relief	85 ± 5 (5)	no relief
5×10^{-4}	33 ± 3 (6)	-	45 ± 5 (5)
10^{-3}	transient relief	-	47 ± 2 (5)

Figures are ± S.E.M.; the number of observations is shown in parentheses. SCh concentration 9×10^{-6}M.

The antidotal action of the hydroxyanilinium compounds diminished sharply with the development of tachyphylaxis to the SCh blockade. Therefore the figures shown in Table 9 were obtained from first and second exposures to SCh, before the development of tachyphylaxis which was observed at or

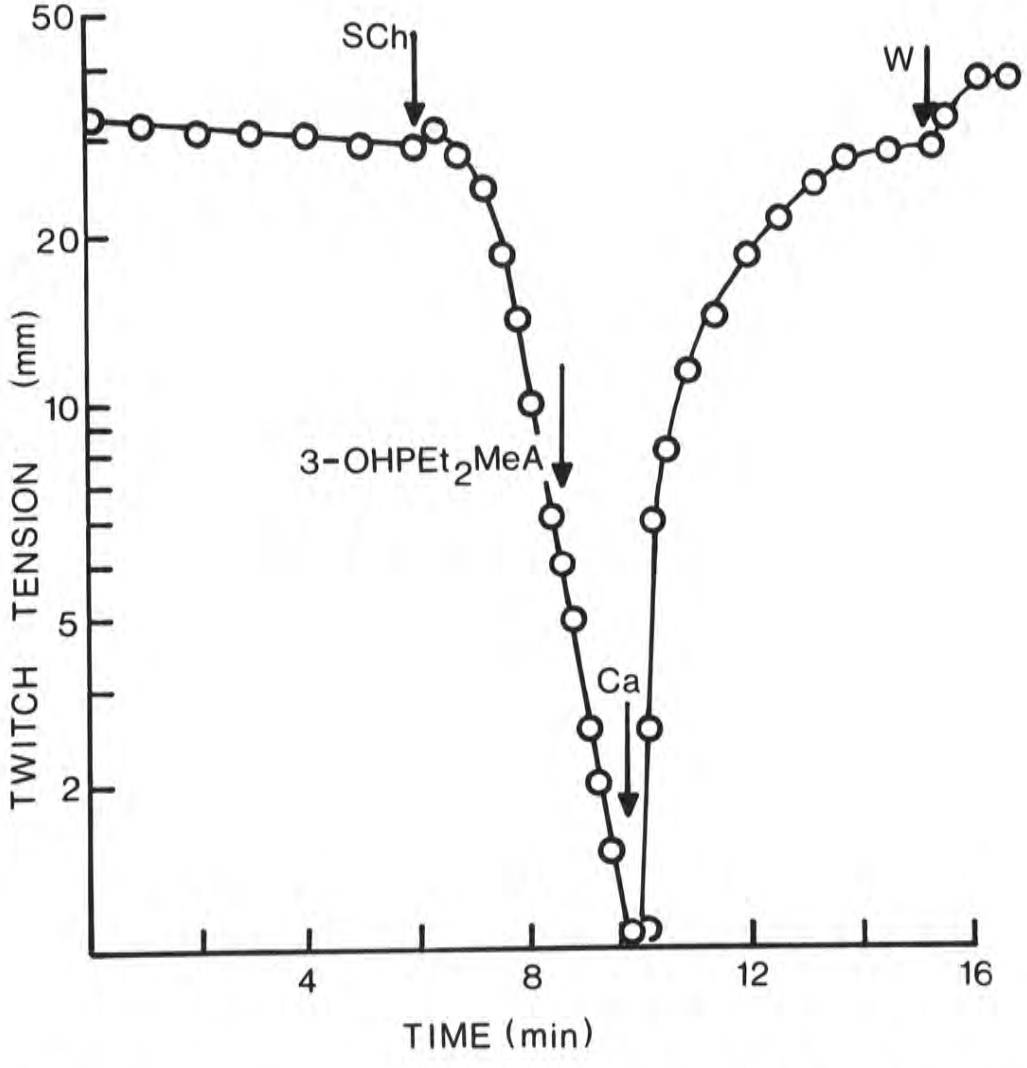


Figure 29: The effect of SCh (9×10^{-6}M) and 3-OHPEt$_2$MeA (10^{-4}M) on partial blockade of the phrenic nerve-diaphragm preparation by 0.15mM Ca^{++}. The drugs were added at the arrows. The external Ca^{++} level was restored to 1.5mM (normal) at Ca. Time to half block ($t_{\frac{1}{2}}$) in low Ca^{++} = 24 minutes; after addition of SCh, $t_{\frac{1}{2}}$ = 0.5 minutes. Semi-logarithmic scale; 25mm twitch tension = 10g.

after the third exposure to SCh.

It was noted earlier that reduction of the external Ca^{++} concentration abolished twitch potentiation by the hydroxyanilinium compounds. The results illustrated in figure 29 demonstrate that antagonism of SCh blockade is also dependent upon the presence of a normal Ca^{++} concentration. SCh (9×10^{-6}M) and 3-OHPEt$_2$MeA (10^{-4}M) were added sequentially to a preparation in which twitch tension was slowly declining due to reduction in Ca^{++} concentration to one-tenth normal (0.15mM). SCh caused a temporary alleviation of the low-Ca^{++} blockade, followed after 1 minute by a greatly increased rate of blockade. 3-OHPEt$_2$MeA added during the course of the SCh-low Ca^{++} blockade did not affect the time course of the exponential decline in tension. However, when twitch tension had fallen to zero, the Ca^{++} level was restored to normal (1.5mM); this rapidly restored twitch tension to 85% of the control level. Similar results were obtained with 3-OHPTEA and edrophonium, except that restitution of the external Ca^{++} achieved only partial alleviation of the SCh blockade, as would be expected from Table 9.

Although only the diethylmethyl hydroxyanilinium compound was found to be an effective SCh antagonist, the converse applied in that SCh antagonized drug-induced facilitation effectively in each case. Figure 22, panels 1 and 3, shows the antagonism of facilitation due to tacrine and edrophonium by SCh at a concentration of 9×10^{-6}M. This concentration of SCh is the minimum dose necessary to produce complete blockade of an untreated

diaphragm (Freeman, 1968a, b). At this concentration antagonism of twitch potentiation is followed by SCh blockade. It was found, however, that 9×10^{-7}M SCh (one-tenth of the blocking dose) was sufficient to abolish twitch potentiation due to tacrine, edrophonium and 3-OHPTEA. 3×10^{-6}M SCh was required to abolish potentiation by 3-OHPEt$_2$MeA (figure 22, panel 8). These findings are consistent with the observation of Standaert and Adams (1965) that post-tetanic repetition is depressed at SCh doses ten times less than those required to depress transmission.

DISCUSSION

Potentiation of the indirectly elicited isometric twitch may be due to facilitation of one or several steps in the process of neuromuscular transmission. Blaber and Karczmar (1967a) have discussed the probability of multiple cholinoceptive sites at the neuromuscular junction, all of which may interact with facilitatory drugs. The sites are considered to be the motor end-plate, acetylcholinesterase and two sites at the nerve terminal, one of which may be the most distal node of Ranvier (Hubbard, Schmidt and Yokota, 1965; Freeman, 1968b).

Three of the four facilitatory compounds in this study have been shown to potentiate both the ACh-induced contraction and the ACh-induced depolarization of the denervated diaphragm and therefore are likely to have a qualitatively similar effect on the postsynaptic membrane. The fourth compound (3-OHPTEA) had a depressant effect at concentrations which produced facilitation of the indirectly elicited twitch in the innervated preparation.

All four facilitatory drugs are AChE inhibitors of various potencies (Ho and Freeman, 1965; Kuperman et al., 1961).

The effects of these compounds on the nerve terminals must now be considered. Many authors have produced evidence of presynaptic effects (Standaert and Riker, 1967; Blaber and Karczmar, 1967a and b); however, neither the site nor the mechanism of these effects has been determined. The present experiments, which demonstrate the sensitivity of twitch potentiation to small changes in divalent cation

concentration, suggest that the unmyelinated nerve terminals, which are the site of ACh release, undergo repetitive activity, either spontaneously or in response to stimulation, in the presence of these drugs (Werner, 1960).

The possibility that such activity may originate in the most distal nodes of Ranvier must also be considered. The Ca^{++}-independent antagonism of SCh blockade by tetraethylammonium and triethylcholine has been previously attributed to a nodal action of these drugs, resulting in prolongation of the action potential invading the terminals and an increase in the safety margin for ACh release in the presence of low Ca^{++} or SCh (Freeman, 1968b).

However, SCh antagonism by the hydroxyanilinium compounds is Ca^{++} dependent, which suggests that their action is largely at the nerve terminal where Ca^{++} plays an important role in transmitter release (for references see Katz and Miledi, 1968). Although these authors obtained their evidence from amphibian preparations, their findings are likely to be valid also for mammalian preparations (Riker and Okamoto, 1969). It may be further suggested that SCh suppression of both drug-induced and post-tetanic potentiation is related to competition between SCh and Ca^{++} in the nerve terminal membrane, with consequent damping of potential oscillations (Freeman, 1968a and b).

Twitch potentiation cannot, however, be equated entirely with the mechanism of SCh antagonism, as is clear from the present investigation and the findings of other

workers (Blaber and Karczmar, 1967a and b). Although tacrine causes twitch potentiation it shows no antagonism of SCh blockade. In addition, it is an anticholinesterase of comparable potency to 3-OHPEt$_2$MeA, and causes sensitization of the ACh receptor. Therefore, other properties must account for SCh antagonism by the hydroxyanilinium compounds. Structurally, tacrine is unrelated to the hydroxyaniliniums. The amino group of tacrine is largely ionized at physiological pH (pK_a = 9.95) (Albert and Goldacre, 1946) but this compound lacks the substituted onium group that appears to be common to SCh antagonists (Freeman, 1968b).

The finding that facilitatory drugs all have some anticholinesterase activity is possibly a function of the structural similarity of all cholinoceptive sites rather than a reflection of a causal relationship.

While the results obtained with the denervated diaphragm preparation show that postsynaptic action, presumably by a process of receptor sensitization, is likely to be a contributing factor in the mechanism of action of the facilitatory drugs studied, the findings of this study favour a predominantly presynaptic action. The high temperature coefficient of facilitation is opposite in sign to the increase in postsynaptic membrane sensitivity which is observed at low temperature. In addition, the effects of variation in the divalent cation concentration of the bathing solution on facilitation in the intact junction are opposite to those found for the denervated receptor.

Appendix 1: Composition of nutrient solutions.

All solutions were aerated with 95% O_2/5% CO_2.

NORMAL NUTRIENT SOLUTION

(used for all preparations except those referred to below)

	Concentration (mM)
NaCl	115
KCl	4.6
$CaCl_2$	1.5 *
$MgSO_4$	1.0
NaH_2PO_4	1.2
$NaHCO_3$	22
glucose	22

pH 7.4 at 30°C

* This level of Ca^{++} is lower than that used by many workers. It is in agreement, however, with the finding of Van Breeman, Daniel and Van Breeman (1966) that, in rat plasma, only 1.5mM Ca^{++} is not bound to albumin.

ISOLATED HEART NUTRIENT SOLUTION

	Concentration (mM)
NaCl	115
KCl	4.6
$CaCl_2$	1.8
$MgSO_4$	1.2
NaH_2PO_4	1.2
$NaHCO_3$	22
glucose	22

TOAD NUTRIENT SOLUTION

	Concentration (mM)
NaCl	96
KCl	3.8
$CaCl_2$	1.5
$MgSO_4$	0.8
NaH_2PO_4	1.0
$NaHCO_3$	18
glucose	18

Appendix 2: Drugs and media used.

Concentrations given in the toxin experiments refer to weights of these salts. The source of supply is indicated in brackets.

acetylcholine chloride (Koch-Light)
adenosine (Koch-Light)
adenosine-5'-triphosphate (Sigma)
l-adrenaline bitartrate (K and K)
atropine sulphate (Macfarlan Smith)
L(+)-ascorbic acid (May and Baker)
bretylium tosylate (Burroughs Wellcome)
carbamylcholine chloride (carbachol) (British Drug Houses)
chloralose (British Drug Houses)
diphenhydramine hydrochloride (Benadryl) (Parke Davis)
fluothane (I.C.I.)
heparin (in normal saline) (Commonwealth Serum Laboratories)
hexamethonium bromide (Koch-Light)
histamine acid phosphate (British Drug Houses)
3-hydroxyphenyldiethylmethylammonium bromide (3-OHPEt$_2$MeA)[1]
3-hydroxyphenyldimethylethylammonium chloride (edrophonium) (Hoffman-La Roche)
3-hydroxyphenyltriethylammonium bromide (3-OHPTEA)[2]
5-hydroxytryptamine (serotonin creatinine sulphate) (Koch-Light)
isoprenaline sulphate (Burroughs Wellcome)
lysergic acid diethylamide (Delysid) (Sandoz)
l-noradrenaline bitartrate (Koch-Light; Winthrop)
pentobarbital (Abbott)

phenoxybenzamine hydrochloride (Smith, Kline and French)

phentolamine (regitine methanesulphonate) (CIBA)

procaine hydrochloride (K and K)

propranolol hydrochloride (Inderal) (I.C.I.)

saxitoxin[3]

succinylchôline chloride (Glaxo-Allenburys)

tetrahydro-4-aminoacridine hydrochloride (tacrine) (H.W. Woods)

tetrodotoxin (crystalline 3x) (Sankyo)

tris (hydroxymethyl) aminomethane hydrochloride (Tris) (Merck)

d-tubocurarine chloride (Baxter-D.H.A.; K and K)

urethane (Koch-Light)

Sephadex G-25
G-75
G-200

Blue dextran 2000

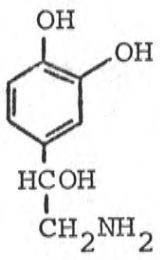
Macrodex (6% dextran, M.W. 70,000 in 0.9% NaCl) (Pharmacia)

Amberlite IRC-50 (Analytical Grade) (British Drug Houses)

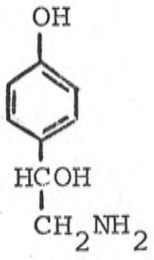
1,2 Synthesized by Mr. D. Amos, Defence Standards Laboratories.

3 Generously supplied by Dr. E.J. Schantz, Fort Detrick, Maryland.

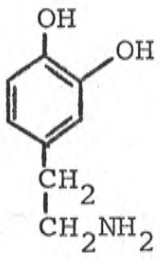
Appendix 3: Formulae of some biologically active compounds which have been isolated from Cnidaria and Mollusca.



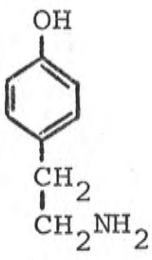
Noradrenaline



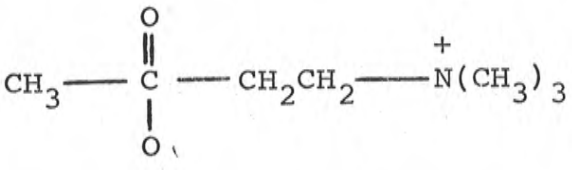
Octopamine



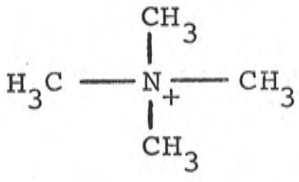
Dopamine



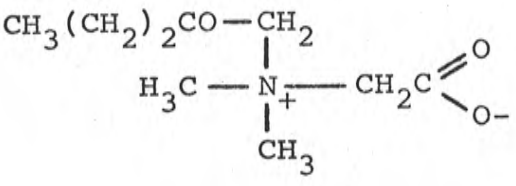
Tyramine



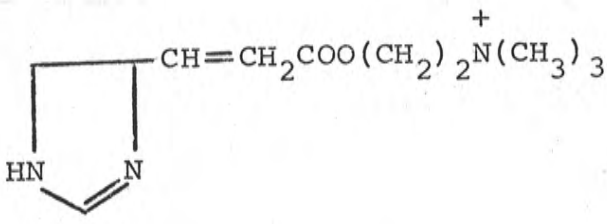
Acetylcholine



Tetramine

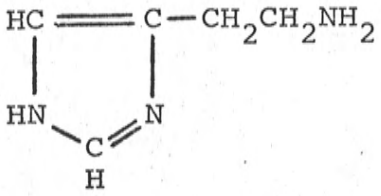


Gamma-butyrobetaine

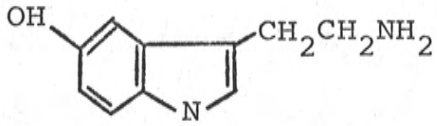


Murexine (urocanylcholine)

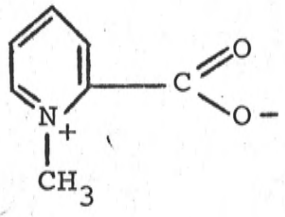
Appendix 3 (continued)



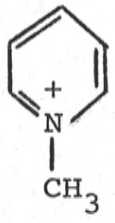
Histamine



5-hydroxytryptamine(serotonin)



Homarine



N-methylpyridinium

Appendix 4: List of publications.

1. FREEMAN, S.E. and TURNER, R.J. (1968) Succinylcholine blockade of the mammalian neuromuscular junction after acetylcholinesterase inhibition with DFP. Life Sciences 7, 875 - 881.

2. FREEMAN, S.E. and TURNER, R.J. (1968) Ionic interactions in acetylcholine contraction of the denervated rat diaphragm. Proc.Intern.Union Physiol.Sci. 7, 430.

3. FREEMAN, S.E. and TURNER, R.J. (1969) A pharmacological study of the toxin of a cnidarian Chironex fleckeri Southcott. Br.J.Pharmac. 35, 510 - 520.

4. FREEMAN, S.E. and TURNER, R.J. (1969) Ionic interactions in acetylcholine contraction of the denervated rat diaphragm. Br.J.Pharmac. 36, 510 - 522.

5. TURNER, R.J. and FREEMAN, S.E. (1969) Effects of Chironex fleckeri toxin on the isolated perfused guinea pig heart. Toxicon, 7, 277 - 286.

6. FREEMAN, S.E. and TURNER, R.J. (1970) Maculotoxin, a potent toxin secreted by Octopus maculosus Hoyle. Toxicol. and Appl. Pharmacol. 16, 681 - 690.

7. FREEMAN, S.E. and TURNER, R.J. (1970) Facilitatory drug action on the isolated phrenic nerve-diaphragm preparation of the rat. J.Pharmac.exp.Ther. 174, 550 - 559.

Appendix 4: (continued)

8. FREEMAN, S.E. and TURNER, R.J. (1970) Cardiovascular effects of toxins isolated from the cnidarian Chironex fleckeri Southcott. Brit.J.Pharmac. (in press).

Reprints of numbers 1, 3-7 are included at the back of the thesis.

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Life Sciences Vol. 7, Part I, pp. 875-881, 1968. Pergamon Press
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SUCCINYLCHOLINE BLOCKADE OF THE MAMMALIAN NEUROMUSCULAR JUNCTION AFTER ACETYLCHOLINESTERASE INHIBITION WITH DFP

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(Received 8 April 1968; in final form 7 June 1968)

Evidence has accumulated in recent years that suggests that succinylcholine (SCh) exerts its characteristic blockade of the mammalian neuromuscular junction at least partly by decreasing the presynaptic release of acetylcholine (ACh). Thus the quantum content of the end-plate potential is reduced[1], post-tetanic repetition is abolished[2], and alteration of the external ionic environment either augments or reduces the rate of onset of blockade, in a manner consistent with a presynaptic mechanism[3]. Further, drugs such as tetraethylammonium and triethylcholine, which increase ACh release, prevent or reverse SCh blockade[4].

Measurement of the amount of ACh released during stimulation in the presence of SCh was carried out with a technique very similar to that of Beani et al[5], in order to test this hypothesis directly.

Methods

The phrenic nerve-diaphragm of the immature guinea pig (300-370 gm) was used. Companion hemidiaphragms were set up in organ baths at 29°C; di-isopropylfluorophosphonate (DFP) at a concentration of 500 µg/ml was used to inhibit acetylcholinesterase. The preparations were treated with the inhibitor for two hours; it was then removed by repeated washing in nutrient solution[3] prior to the collection of ACh. Stimulation at a frequency of 20/sec for 10 min followed by 10 min rest was used as the collection period; resting release of ACh was estimated from the solution bathing the preparations for 10 min at rest. SCh (9×10^{-6} M) was added to the bath prior to the second period of stimulation. Acetylcholine collected was assayed using the isolated ileum of the guinea pig[5]. Preliminary experiments showed that SCh at the concentration used did not alter the dose-response curve of the assay. Isometric tension was recorded using Statham UC2 transducing cells and a

Beckman type R Dynograph recorder.

Results

The results of these experiments are illustrated in Fig. 1. It may be seen that this concentration of SCh did not significantly affect the release of ACh under our experimental conditions.

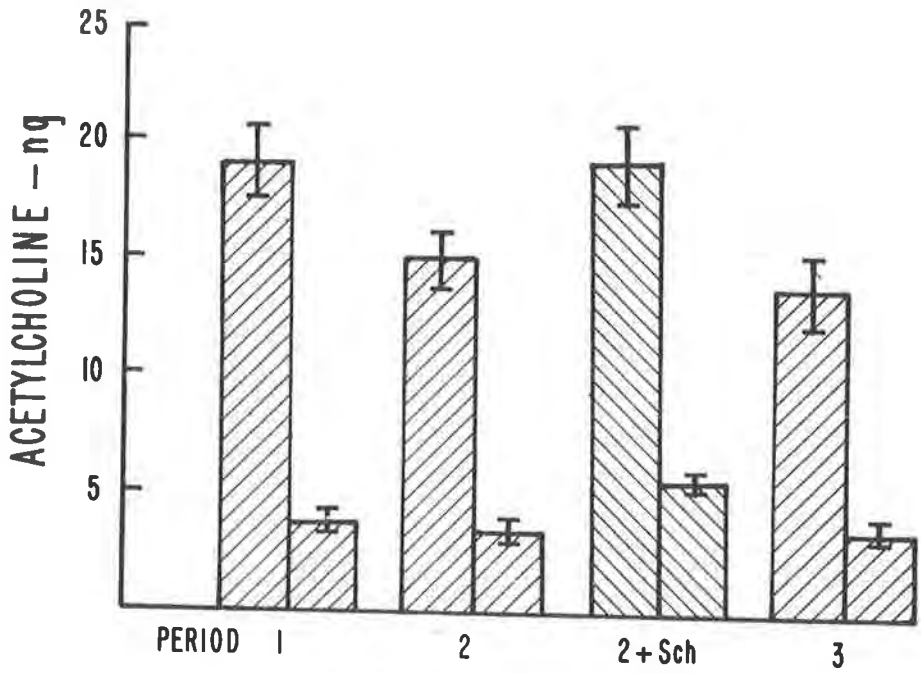

FIG. 1

The release of acetylcholine in nanogram during successive periods of stimulation at 20 cps for 10 min followed by 10 min rest. Resting release during a subsequent 10 min period is seen to be approximately 5 times less than during stimulation. Acetylcholine release in the presence of succinylcholine (9×10^{-6}M) is not significantly different from the control. Vertical bars indicate $\pm$ S.E. of the mean. Temperature 29°C.

An explanation of this apparently anomalous result was evident when a study was made of the nature of SCh blockade in the DFP-poisoned preparation. Experiments were carried out with companion hemidiaphragm preparations. One was poisoned with DFP in the usual way, the other was maintained in the normal solution for the same period of time. It was found that SCh at a concentration of 3×10^{-6}M was sufficient to block completely the control preparation which was stimulated at a frequency of 0.1 per sec. However, a

concentration of 9×10^{-6} M SCh was needed for complete blockade after DFP treatment. Further, the form of the curve of twitch tension against time differed in the poisoned preparation. In the control preparation blockade occurred at an approximately exponential rate after a latent period of approximately 6 min. The same concentration of SCh did not completely block the DFP-treated preparation; the block occurred exponentially after a much shorter latent period. At 3×10^{-6} M SCh the latent period in the DFP-treated preparation was 2.5 ± 0.6 min (S.E. of 5 observations) and the time to half-blockade was 8.8 ± 1.2 min. In the untreated preparation the latent period was 6.0 ± 0.7 min (S.E. of 6 observations) and the time to half-blockade was 1.3 ± 0.2 min. The differences in latency and time to half block were significant at $P < 0.006$. It was noted that the semilogarithmic plot of twitch tension against time (Fig. 2) could not be as neatly divided into a latent period followed by an exponential decline in tension as was found previously for the rat diaphragm [3, 4].

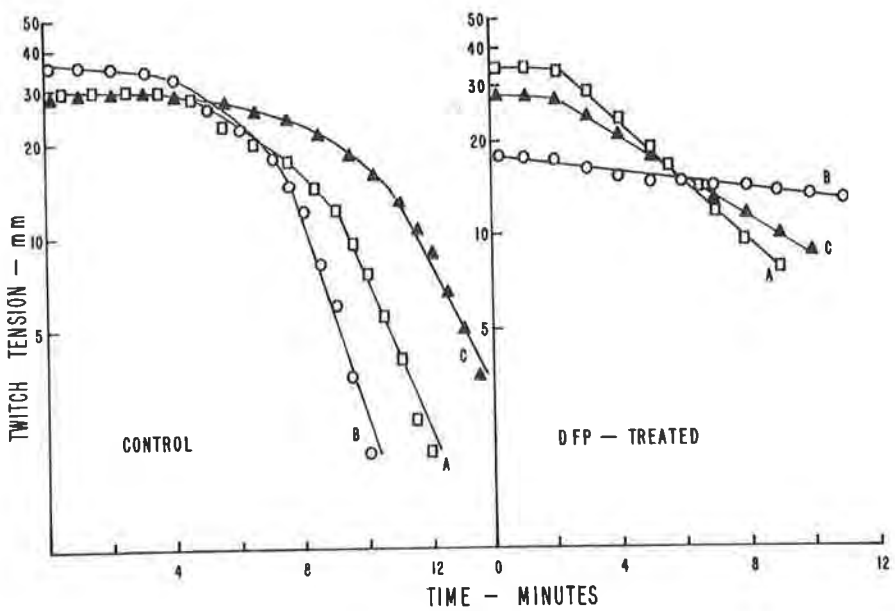

FIG. 2

Semilogarithmic plots of the decline in twitch tension during successive SCh blocks of paired diaphragms. SCh 3×10^{-6} M. In both instances the second block B was carried out at 20.5°C, blocks A and C were carried out at 29.5°C. 27 mm tension = 10 g.

This may be related to the greater mean thickness of the muscle (1.3 mm compared with 0.64 mm in the rat) and also to the fact that one side of the diaphragm tended to cling to the rib-cage in a rather variable way. These two facts introduce uncertainties due to variation in rates of diffusion; however comparisons between paired preparations remain meaningful.

It was shown previously[3] that SCh blockade of the rat diaphragm proceeded more rapidly at 20°C than at 29°C. This is evident also in the guinea pig preparation (Fig. 2). This figure shows the increased rate of block at 20.5°C compared with prior and subsequent blocks at 29.5°C; a slight but consistent development of tachyphylaxis in the third block is also evident. Fig. 2 also shows a similar experiment carried out on the companion hemi-diaphragm after DFP treatment. It is clear that the second, low temperature block proceeded at a far slower rate than prior and subsequent blocks at 29.5°C. Again tachyphylaxis was found to develop to repeated SCh blockade.

The inference that blockade differed in mechanism after DFP treatment was confirmed in further experiments. Fig. 3 illustrates the effects of alterations of the external ionic environment during the course of blockade in both the treated and control preparations.

As was found in the rat diaphragm the time to half-blockade was reduced 5 fold by doubling the external K^+ concentration (9.2 mM). Raising the Mg^{++} concentration from 1 to 3 mM brought about a 60 per cent reduction in the time to half block. Since the rate of blockade of the preparation is normally very rapid these effects were most easily demonstrated in preparations which had developed a slight degree of tachyphylaxis to SCh, and where blockade was slower than usual. Doubling the external Ca^{++} level (3.0 mM Ca^{++}) alleviated an existing block and restored twitch tension to an average of 75 per cent of the original level. It was further noted that exposure to high external Ca^{++} caused a permanent increase in the time to half blockade upon subsequent addition of SCh.

After DFP treatment high Ca^{++} solution doubled the time to half block; in no experiment was Ca^{++} elevation able to halt or to alleviate blockade. Doubling the external K^+ level caused a consistent slowing of SCh blockade after DFP treatment; the time to half block was increased 4 fold. The effect of raising the external Mg^{++} level to 3 mM was somewhat similar to the effect

in the untreated preparation, i.e. the time to half block was reduced by half.

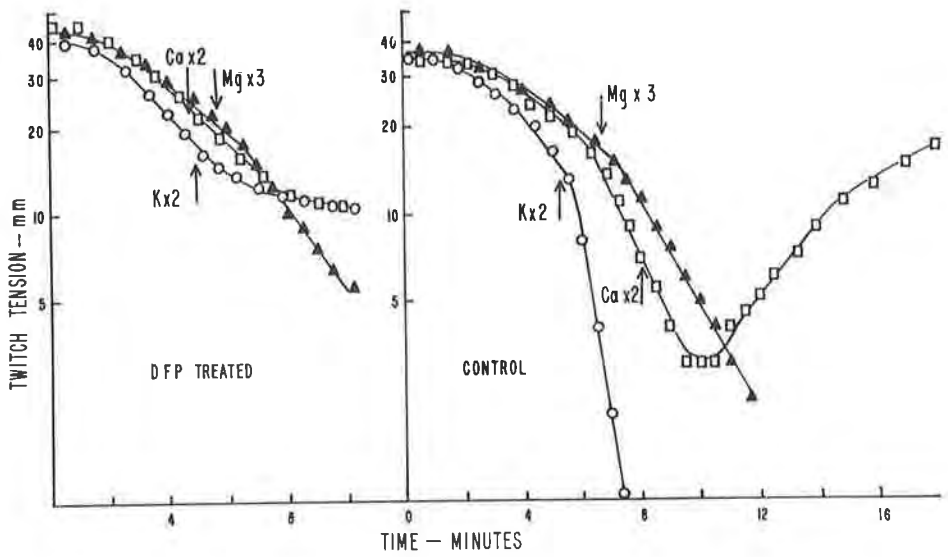

FIG. 3

The effect of alteration of the ionic environment on the rate of SCh blockade. K, Ca and Mg levels were raised at arrows. Semilogarithmic plot; 27 mm tension = 10 g.

It will be noted from Fig. 3 that the ionic effects were only evident after a delay of 1-2 min. It is likely that this reflects, at least in part, the slowness of diffusion into these preparations.

Interactions between d-tubocurarine (dTC) and SCh were also different after DFP treatment. In distinction to the finding with SCh, blockade by dTC (1.3×10^{-6}M) was approximately 50 per cent faster in the DFP treated diaphragm. SCh (3×10^{-6}M) stopped the progress of a dTC block in the untreated preparation, but did not reverse it. After treatment with DFP, SCh caused a barely susceptible slowing of the dTC blockade.

It was shown previously[4, 6] that the dicarboxamide, methoxy-ambenonium, completely reversed SCh blockade of the rat phrenic-nerve diaphragm preparation. This has been confirmed in the guinea pig. It was noted however that after treatment with DFP, methoxyambenonium increased the rate of SCh blockade of the junction.

It may be inferred from these results that blockade of the untreated

guinea pig diaphragm at 29°C may be likened to Phase 1 block (for references see [7]), whereas after DFP treatment SCh blockade resembles Phase II blockade. It has been suggested that Phase I blockade is a depolarizing blockade, which after prolonged exposure to the depolarizing drug changes in character, and becomes "non-depolarizing". It is assumed that Phase II blockade resembles, but is not identical with competitive block due to dTC or its congeners. As such it is relieved by neostigmine, high K^+ solutions and reduction in temperature.

The hypothesis is put forward that Phase I blockade results from a reduction in the presynaptic output of ACh, as well as from depolarization of the postsynaptic membrane. Such a hypothesis is supported by the ionic evidence[3], as well as by the effects of temperature change and drug antagonism[4]. Prolonged exposure to a depolarizing drug, whether it be SCh or endogenous ACh, leads to changes in the junction which are apparent as Phase II blockade. The high level of DFP which is necessary to inhibit AChE[5] may promote Phase II blockade by some mechanism additional to the inhibition of this enzyme. It is likely however that blockade after DFP treatment lacks entirely the presynaptic component, and results from changes in the postsynaptic membrane due to prolonged depolarization and subsequent desensitization. These changes result in an altered configuration of the membrane whereby the drug-receptor complex resembles that due to dTC.

A note of caution may be sounded to all those who attempt a pharmacological dissection (as it were) of the neuromuscular junction. The manipulation may produce unexpected changes.

Acknowledgements

We wish to thank Glaxo-Allenburys (Australia) Pty. Ltd. for a gift of succinylcholine, and the Sterling-Winthrop Research Institute for a gift of methoxyambenonium.

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A PHARMACOLOGICAL STUDY OF THE TOXIN OF A CNIDARIAN, *CHIRONEX FLECKERI* SOUTHCOTT

BY

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Reprinted from BRITISH JOURNAL OF PHARMACOLOGY, *March*, 1969, *vol.* 35, *No.* 3, *p.* 510

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Br. J. Pharmac. (1969), **35**, 510–520.

A pharmacological study of the toxin of a Cnidarian, *Chironex fleckeri* Southcott

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1. A study has been made of the pharmacological actions of toxic preparations obtained from the box jellyfish *Chironex fleckeri* Southcott. Two toxin preparations were used. One was a tentacle extract which was partially purified by Sephadex gel filtration; the second was obtained by a process analogous to snake milking, and is probably similar in composition to the material injected into victims.

2. All preparations were extremely toxic; death in animals, following minimally lethal doses, occurred in minutes. Respiratory arrest of central origin appeared to be the terminal event in all species tested. This was accompanied by marked signs of cardiotoxicity. The heart was slowed, irregular, and showed varying degrees of conduction delay. Terminally it showed atrioventricular block.

3. Blood pressure changes were biphasic. An initial rise in carotid pressure was followed by a profound fall; a second rise to an above normal level frequently followed this. These blood pressure oscillations were damped down by prior treatment with hexamethonium but the hypertensive response remained.

4. Blood samples taken before terminal apnoea showed a variable degree of haemolysis and a raised K^+ level.

5. Experiments with isolated organ preparations suggested that the toxin had a non-specific lytic effect on cells, but did not contain pharmacologically active substances of small molecular weight such as 5-hydroxytryptamine.

6. It is suggested that the toxin(s) act by altering membrane permeability; the signs at death may reflect the sensitivity of the target organs to such a change.

The Cnidarian *Chironex fleckeri* Southcott 1956, popularly known as the box jellyfish, or sea wasp, has been responsible for some sixty deaths among swimmers in Australian tropical waters (Barnes, 1967; Cleland and Southcott, 1965). When it is considered that most stings from *C. fleckeri* and related cubomedusae are non-fatal, and produce minor (though extremely painful) injuries, it will be appreciated

that these species constitute a considerable hazard to public health in Northern Australia. The medical aspects of fatal and non-fatal stings have been well documented by Barnes (1966); he has also reported on the zoology and ecology of the species.

The nature of the toxic material which produces the lash-like lesions and death is not known, nor has its pharmacology been studied. The present communication describes an investigation of pharmacological aspects of the toxin; so far, no specific treatment of the stinging can be indicated. The biochemistry of the toxic material will be described elsewhere.

Methods

Collection and preparation of toxin

Live specimens of *C. fleckeri* were caught in North Queensland waters in midsummer by Dr. J. H. Barnes. They were placed in sea water containers in a cold room at approximately 8° C for from 3–24 hr. They were then carefully lifted from the sea water by grasping the apex of the umbrella and the tentacles were cut off at each pedalium and allowed to fall into a container of liquid nitrogen. They were freighted to the laboratory in this container.

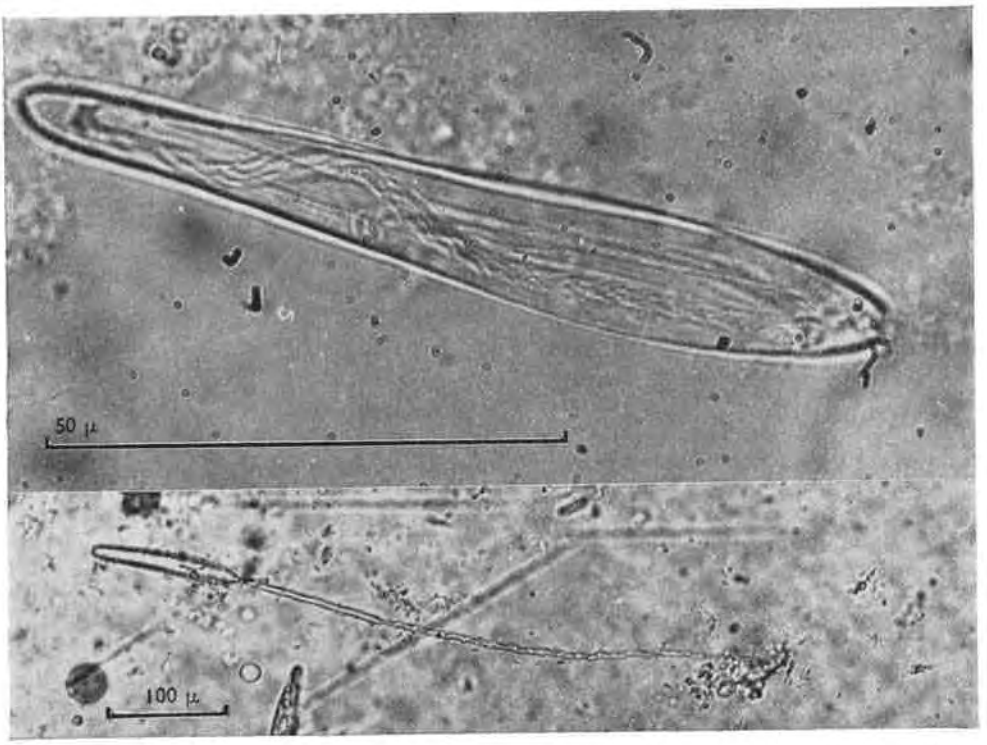

FIG. 1. Nematocysts isolated from frozen *Chironex* tentacle. These are the poison-containing microbasic mastigophore; tentacles contain several other types (Barnes, 1967). The upper picture shows the undischarged capsule; most were in this state in the frozen tentacle. The lower picture shows a discharged capsule with the long lash still intact.

The preliminary cooling of the animals before freezing seemed to reduce nematocyst discharge to a negligible amount, because nematocysts isolated from the tentacles were largely undischarged (Fig. 1); this finding was confirmed by Dr. Barnes who examined formalin-fixed sections.

Toxin extracts were prepared by mincing the thawed tentacle in 7% sucrose at 0° C. Robson (1953) found that sucrose reduced nematocyst discharge in *Corynactis*. Two volumes of sucrose solution were added to one of tentacle, and the resulting suspension was strained through a stainless steel mesh to remove coarse particulate matter and centrifuged for 5 min to throw down the nematocysts. All operations were carried out at approximately 4° C.

It was hoped that the toxic material might be retained in the undischarged capsules. It was found, however, that the greater part of the toxicity was present in the supernatant. Grinding the nematocyst preparation in a glass homogenizer or sonic disintegration did not increase the toxicity of this fraction. It must be assumed that the toxin had leaked out of the apparently intact nematocysts during preparation.

The sucrose solution of toxins was partially purified by gel filtration through a Sephadex G-75 column at 5° C; the column was adjusted to pH 6.3 with Tris-maleate buffer (Lane, 1967). The lethality of the eluted fractions was tested by injection into the tail vein of white mice weighing approximately 20 g. Peak lethality appeared in fractions eluted from the column a few ml. after the passage of the void volume, which corresponded to a molecular weight greater than bovine serum albumin. However, lethality was spread through the column down to an elution volume consistent with a molecular weight of approximately 8,000. Although the sucrose extract was extremely labile at room temperature, it did not lose lethality for upwards of 24 hr after gel filtration. Solutions after gel filtration were colourless or faintly opalescent; the material was non-dialysable. A fuller description of the biochemistry of the toxin will be published separately.

Part of the pharmacological investigation was repeated using a sample of toxin obtained from Dr. Barnes, who collected it by a procedure analogous to snake milking. Tentacles applied to one side of an amniotic membrane were stimulated electrically; on nematocyst discharge the injector threads penetrated the membrane and deposited toxin (Barnes, 1967).

Pharmacological testing of toxin

The effect of the toxin on various physiological parameters was tested using New Zealand rabbits (2–3 kg) or Wistar rats (250–300 g). The rabbits were anaesthetized by injecting a 20% solution of urethane into the ear vein; the rats received pentobarbital 40–50 mg/kg by the intraperitoneal route. Arterial and venous pressures were monitored using Statham pressure transducers and a Beckman type R dynograph recorder. The venous catheter was placed in the jugular vein and passed down to the level of the right atrium. Electrocardiograms were recorded from leads I, II and III; heart rate was recorded with a cardiotachometer. Abdominal respiratory movements were recorded using a colloidal carbon-in-rubber pneumograph transducer placed just below the xiphisternum; body temperature was monitored with a rectal thermistor.

In other experiments the phrenic nerve of the rabbit was exposed in the neck and platinum electrodes placed on it. A myograph needle was placed in the diaphragm muscle and the respiratory activity of both nerve and muscle was displayed on a two channel oscilloscope and photographed on moving film.

The effect of the toxin on the release of vaso-active substances from the skin was tested using a technique similar to that of Rocha e Silva & Rosenthal (1961). An air pocket was formed under the dorsal skin of an anaesthetized rat, the animal was hung upside down by its four paws and successive 5 ml. washings of Tyrode solution were introduced into the air pocket. Toxin, equivalent to approximately five lethal doses if given intravenously, was injected intradermally into the skin of the pouch after the first control wash. The animal was finally killed by injection of the toxin into the tail vein.

The effect of the toxin was also tested on the isolated phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946), the isolated guinea-pig ileum and the isolated guinea-pig trachea (Jamieson, 1962). Histamine release was determined using a mast cell preparation obtained by perfusing the abdominal cavity of rats with 20 ml. of physiological saline (Rothschild, 1962). Histamine was assayed using the isolated guinea-pig ileum. The specificity of the histamine-induced contraction was determined by blocking the response with diphenhydramine.

Plasma electrolytes were determined in heparinized samples using an EEL flame photometer.

Results

Effects in mice

The lethality of the toxic effects in mice, after intravenous infection, was determined in an effort to arrive at a " mouse unit ". It was found that 0.1 ml. of a 5,000 fold dilution of the tentacle extract would kill a 20 g mouse in less than 2 min. The extreme lability of the material, even at 0° C, however, made the determination of the unit in the crude extract hazardous. Further, the dose-mortality curve was extremely steep. However, fractions from the Sephadex column were standardized in this way, as was also the relatively stable " milked " toxin obtained from Dr. Barnes.

Mice rarely died earlier than 60 sec after a fatal injection and no deaths later than 22 min were recorded. The progress of death was similar to that described by Barnes (1967). The animals became lethargic and ataxic. Respirations appeared forced and irregular; before death, the animals convulsed briefly but sometimes quite violently. At autopsy the heart was frequently found to be beating with a 3 : 1 atrioventricular block. There was marked venous engorgement. Characteristically, the lungs were stained a reddish-orange; however, both the intensity of staining and the colour were variable. It was impossible to correlate the lung changes with time to death or with the intensity of the terminal convulsions. No other organs showed any macroscopically evident pathology, other than that referable to venous engorgement.

The symptoms were identical whether the mice were injected with the crude extract, with fractions from the Sephadex column or with the " milked " venom.

Effects in rabbits

Arterial and venous pressure, respiratory rate and depth and electrocardiograms were recorded in anaesthetized rabbits. Fractions from the Sephadex column were used exclusively in these experiments. The quantity of toxin required to kill the rabbit was extremely variable, ranging from 10 to 160 " mouse units ". The variation could not be correlated with the number of non-lethal injections preceding the fatal dose, so that it did not seem to arise from a cumulative effect of the toxin. Effects following lethal and non-lethal doses of toxin were recorded; in general death followed a similar pattern to that seen in mice, except that the anaesthetized animals did not convulse. Autopsy findings were also similar to those in the mouse. The shortest time to death was 90 sec; the longest was 6 min. Rabbits surviving longer than this showed virtually complete recovery of all parameters monitored during the next 5 min.

Blood pressure changes. Effective doses of toxin invariably caused a biphasic change in the carotid arterial pressure. The resting arterial pressure of twelve rabbits varied between 100 and 120 mm Hg; following injection of the toxin it rose by 25–60 mm Hg. Thereafter the arterial pressure fell, only to rise again some 60–90 sec later. There was a rise in venous pressure concurrent with the blood pressure fall. Resting venous pressure varied between zero and -7 cm H_2O, and changes following toxin injection were opposite in sign to arterial pressure changes. The maximum venous pressure increase, noted immediately before respiratory arrest, was 12.5 cm H_2O; the arterial pressure was then 25 mm Hg.

Blood pressure changes can best be illustrated by describing the effects of a near-lethal dose of toxin. Following the injection of toxin the arterial pressure rose from 115 to 140 mm Hg over a period of 8 sec. There was no change in venous pressure. At this point the pulse pressure declined and the electrocardiogram (e.c.g.) showed some extrasystoles and T wave inversion. The arterial pressure then fell, over a period of 20 sec, to 50 mm Hg concurrent with a rise of 4 cm H_2O in the venous pressure. The arterial pressure had fallen to 25 mm Hg, 90 sec after the injection, and the venous pressure had risen to 7 cm H_2O. At this point the animal ceased breathing for 9 sec and the e.c.g. showed T wave inversion and a 2:1 block. Respiration recommenced spontaneously at 99 sec followed by a slow increase in arterial pressure (which showed good pulse amplitude) to 100 mm Hg. At this point respirations again ceased for 10 sec although arterial pressure continued to rise to a plateau of 190 mm Hg. The e.c.g. showed gross T wave abnormality but no conduction block. There was then a slow recovery of all parameters over a further period of 5 min.

These arterial pressure oscillations were a characteristic effect of the toxin. One rabbit showed four such oscillations of diminishing amplitude before death during a hypotensive phase some 5 min after the toxin injection.

Electrocardiographic changes. The resting heart rate of the rabbit under urethane anaesthesia varied between 260 and 340 beats/min. This rate invariably declined at the peak of the first hypertensive response, coincident with the onset of a variable amount of cardiac irregularity. At this point the pulse pressure, which was normally 25–30 mm Hg, fell to as low as 8 mm Hg. It may be assumed that there was a concurrent fall in cardiac output. Individual e.c.g. tracings showed a variable change in T wave form at this point. Fig. 2 shows a characteristic record; about

20 sec after the toxin injection there was an increase in the T-P interval and a short period of gross T wave enlargement. As the arterial pressure declined the heart rate fell to 100–150 beats/min. The e.c.g. at this time frequently showed T wave inversion, conduction delay and irregular 2 : 1 atrioventricular block. It was characteristic that the second blood pressure rise was associated with a dramatic improvement in pulse pressure to 40–50 mm Hg, but with little increase in heart rate. Apparently the cardiac output increased with the improved regularity of the heart, although the e.c.g. was still abnormal. When lethal doses were given (as in Fig. 2) the e.c.g. abnormalities progressed and coincident with respiratory arrest the heart typically showed abnormal ventricular complexes after each third atrial beat. Individual experiments often showed bizarre variations on this general pattern. The heart rate was occasionally seen to decrease and increase several times before death. Periods of nearly normal cardiac activity were sometimes interpolated between chaotically irregular e.c.g. patterns.

Toxin effects after ganglionic blockade. An attempt was made to determine whether the blood pressure changes seen after toxin injection were central or peripheral in origin, by blocking the autonomic ganglia with hexamethonium.

Changes in the physiological parameters measured previously were monitored in the rabbit before and following ganglionic blockade with hexamethonium. The injection of hexamethonium 2.5 mg/kg reduced the arterial blood pressure by 30–45 mm Hg. Heart rate was unchanged; the respiratory rate tended to increase. The

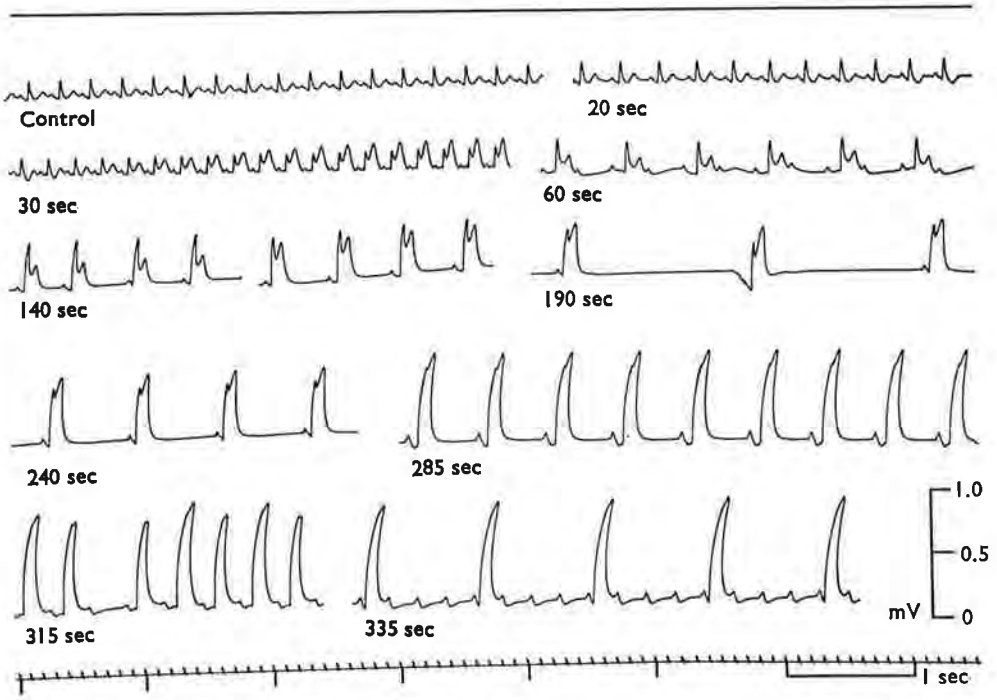

FIG. 2. Electrocardiogram changes following toxin injection. The first panel shows a segment of e.c.g. before injection, heart rate 260/min. There is an increased T-P interval and bradycardia 20 sec post-injection. T-wave enlargement is seen after 30 sec. At 60 sec there is 2:1 atrioventricular block. Between 140 and 190 sec the animal's respirations became irregular and gasping. At 315 sec the heart showed irregular atrioventricular block; the animal ceased to breathe at 320 sec.

most obvious effect of hexamethonium treatment was to damp down the arterial pressure oscillations normally seen after the toxin. The first blood pressure rise occurred some 30–40 sec after toxin injection, compared with 10 sec in the controls. Further, it was maintained for longer, and the subsequent hypotensive episode was less intense. In no instance was a second blood pressure rise seen. Cardiac and respiratory abnormalities developed in the usual way.

Respiratory changes. The respiratory rate of the anaesthetized rabbit showed a variation ranging from 50 to 120 respirations/min. The toxin did not alter respiratory rate or excursion until after the first hypertensive episode, when arterial pressure was falling sharply. At this time, provided the dose of toxin was sufficient, gasping respirations and a period of apnoea were found. The occurrence of apnoea could not be related to the arterial pressure fall in any simple way, because it occurred with pressures ranging from 20 to 100 mm Hg; however, terminal respiratory failure always occurred during a period of acute hypotension. The duration of apnoea was usually 9–10 sec, although one rabbit recovered temporarily after 60 sec of apnoea. During recovery (whether it was permanent or not) the respiratory rate usually, but not invariably, rose—sometimes to twice the resting level. A second short period of apnoea was seen in four instances during the rise in arterial pressure associated with the second hypertensive episode. Thereafter the animal either recovered, or respirations became slower, gasping and ceased.

The results obtained in the rabbit following injections of material obtained by Sephadex filtration were confirmed when samples of Dr. Barnes " milked " venom became available. The pattern of events following near-lethal and lethal injections showed no significant difference from the preceding results. It was noted that an injection of 10 mouse units was sufficient to produce blood pressure, e.c.g. and respiratory changes closely similar to those reported above. A dose of 100 mouse units proved fatal; respiratory failure occurred 5 min after injection into the marginal ear vein.

Effect of vagotomy. Bilateral cervical vagotomy reduced the resting respiratory rate of the rabbit from 90/min to 70/min. Two injections of toxin (one non-lethal, one lethal) given after vagotomy produced a similar pattern of results to those described above except that at no time was there an increase in respiratory rate. The rate fell during the hypotensive episode to 42/min. This was followed by gasping and respirations ceased following a very prolonged inspiration.

Effect on phrenic nerve activity. Respiratory changes were further investigated by monitoring simultaneously the impulses passing down the phrenic nerve and the output of a myograph needle placed in the diaphragm muscle. Fig. 3 shows the results of a typical experiment. Panel 1 illustrates the electrical activity associated with one normal respiration. Inspiration lasted for approximately 0.3 sec. The respiratory pause was 0.55 sec. Panel 2 shows the increased respiratory rate after a non-lethal dose of toxin. The duration of inspiration was unchanged; the increased rate was due to a decrease in the respiratory pause. Subsequent panels illustrate the effects of a lethal dose of toxin. Gasping, due to a lengthening of inspiration at the expense of expiration, is shown in Panel 4. At this time respiration appeared to be entirely diaphragmatic and, as is confirmed by the electrical record, there was an augmented force of contraction. Panel 5 illustrates the terminal gasp, before apnoea and death.

The records show that neuromuscular transmission remained effective until death. This was confirmed by placing stimulating electrodes on the nerve *post-mortem*, when it was possible to elicit a vigorous diaphragmatic twitch. It is clear that the respiratory centre remained capable of initiating respiratory signals until the final respiration.

Effects in rats

The findings recorded in the rabbit were confirmed when the same physiological parameters were monitored in the rat. Death followed the injection into the tail vein of 2–4 mouse units of toxin. The time to death varied from 2 to 10 min.

Cardiovascular changes. The resting carotid arterial pressure varied from 155 to 165 mm Hg, pulse pressure was approximately 35 mm Hg and venous pressure (at the level of the right atrium) varied from zero to 2 cm H_2O. The heart rate varied from 390–420 beats/min.

Following injection of toxin the arterial pressure showed a biphasic response similar to that seen in the rabbit. In 10–15 sec there was a rise to 180–200 mm Hg, without change in venous pressure. This was followed by gross T wave enlargement in the e.c.g. The heart rate then slowed, pulse pressure fell and there was a phase of T wave inversion. One animal showed a second transitory rise in arterial pressure, similar to that seen in the rabbit. The rat differed from the rabbit in that there was no rise in venous pressure coincident with the arterial pressure fall. After respiratory failure the e.c.g. usually showed atrioventricular block and large ventricular complexes.

Respiratory changes. Rats showed increases in respiratory rate after toxin injection, gasping and periods of apnoea similar to that seen in the rabbit.

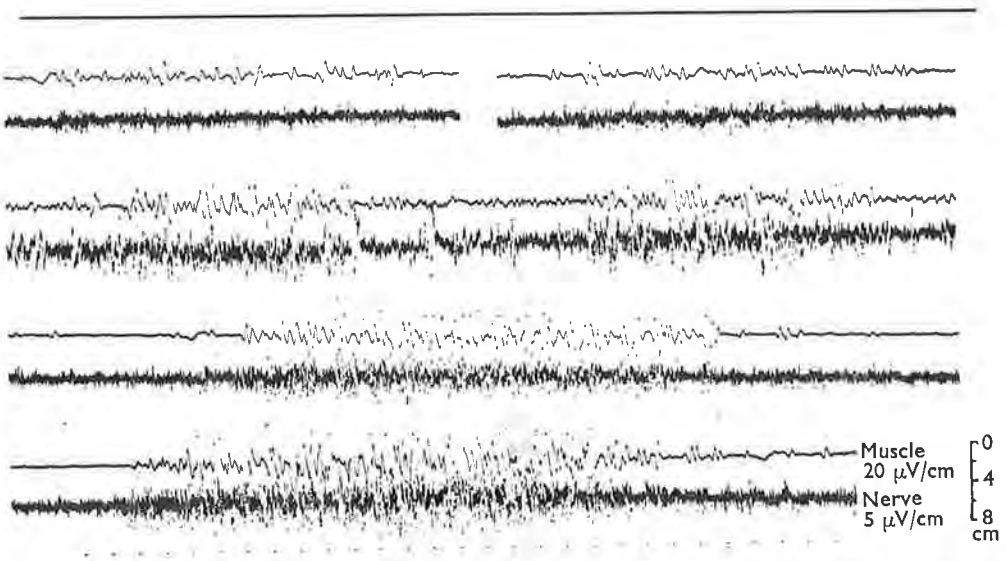

FIG. 3. Phrenic nerve and diaphragm activity following toxin injection. Upper trace shows record from myograph needle in the diaphragm, lower trace shows phrenic impulses. Panel 1 shows a control respiration. Panel 2 shows increased phrenic activity after a non-lethal toxin injection. Panel 3 shows irregular, gasping respirations after a lethal dose. Panels 4 and 5 show the exaggerated activity associated with slow, gasping respirations. Panel 5 shows the animal's terminal gasp, which was followed by respiratory silence. Time marker equals 0.02 sec.

Plasma electrolyte changes. The e.c.g. abnormalities, which typically included T wave elevation followed by inversion, bradycardia and conduction delay, were consistent with hypoxia and/or a release of K^+, followed by an extracellular accumulation of this ion. Consequently blood samples were taken from both rabbits and rats immediately before respiratory arrest. There was a variable amount of haemolysis of the heparinized samples, which could be correlated with the plasma K^+ level. Terminal K^+ levels ranged from 5.8 mM/l. to 19.2 mM/l. No significant change in plasma Na^+ was noted.

It was found that the degree of staining of the lungs seen at autopsy could also be correlated with the degree of haemolysis.

Effects in isolated organs

The observations obtained on the intact animals were extended by the use of isolated organ preparations to find out if part of the clinical picture was due to the presence of pharmacologically active substances of low molecular weight. The long-lasting wheals following stingings that have been described by Southcott (1959) and Barnes (1967) could possibly be related to the injection or release of histamine and/or bradykinin. The painful character of the stings also suggests the possible presence of 5-hydroxytryptamine or acetylcholine. Experiments were carried out using crude tentacle extract, Sephadex eluates and " milked " toxin. The Sephadex eluates were included for comparison because the filtration process would have removed small molecules.

Isolated guinea-pig ileum. All three toxin preparations consistently produced a slow contraction of the guinea-pig ileum. A second dose of toxin after washing the preparation for 15 min produced a much smaller contraction ; it was frequently impossible to obtain a third contraction. Prior exposure of the preparation to the toxin markedly reduced its sensitivity to 5-hydroxytryptamine or histamine. High doses of toxin (2–3 mouse units/ml.) were necessary to produce a contraction.

Isolated guinea-pig trachea. Similar concentrations of toxin also produced slow contractions of the isolated trachea. These were small in amplitude and could be obtained only once or twice in any trachea. They appeared to be blocked by diphenhydramine 2×10^{-8} g/ml., but the difficulty of obtaining repeated responses to the toxin made assay experiments impossible.

Histamine release from mast cells. Toxin doses ranging from 1 to 5 mouse units/ml. brought about some histamine release from mast cell preparations. Sephadex fractions were active in this respect, although less so than crude tentacle extracts. It was noted, however, on microscopic examination of the mast cell preparation that histamine release could be correlated with cell damage, suggesting that the toxin may have a non-specific lytic action. This may also account for its action on the smooth muscle preparations.

Histamine release from a skin pouch. An air pocket was made on the dorsal skin of the rat. Washings of the pocket were made before and after the intradermal injection of 5 times the lethal dose for the rat. All washings were found to contain histamine but in no experiment was the histamine content increased by treatment with the toxin.

Phrenic nerve-diaphragm preparation. Because of the occurrence of respiratory failure, a number of experiments were carried out to test the effect of the various

toxin preparations on the diaphragm preparation. The results were similar in all cases. Doses of toxin equivalent to 10 mouse units/ml. increased the base line tension of the preparation for a period of approximately 10 min. Thereafter there was a slight decline in twitch tension following direct or indirect stimulation. This effect could not be reversed by washing, but did not alter the response of the preparation to succinylcholine or (+)-tubocurarine. Further, the toxin did not affect the response of a denervated diaphragm to acetylcholine, nor was the rate of spontaneous fibrillation of the diaphragm altered. It appears therefore to lack any specific anticholinergic effect.

Discussion

The similarity of the pharmacological effects produced by either the crude tentacle extract, the Sephadex eluate or the " milked " venom suggests that the same toxic principle was present in each preparation. However, there is no evidence to decide whether one toxin or a family of molecules is involved. The possibility that Sephadex filtration might remove pharmacologically active substances of small molecular weight was covered by the use of the amnion-milked preparation. Substances such as 5-hydroxytryptamine, bradykinin, etc., seem to be either absent or present in insignificant amounts. The toxic material is obviously extremely potent ; it will be of considerable interest to investigate its biochemistry further.

The cause of death following injection of the toxin appears to be due to respiratory arrest and cardiotoxicity. Respiratory arrest seems to be central in origin, as is shown in Fig. 3 and the finding that stimulation of the phrenic nerve *post mortem* elicited a strong diaphragmatic twitch. This was confirmed in studies using the isolated phrenic nerve-diaphragm preparation. The cardiotoxicity of the toxin was chiefly manifested as an interference with repolarization and conduction. The heart was slowed, irregular and showed varying degrees of conduction delay, terminating in atrioventricular block.

The finding that the toxin is haemolytic confirms the observation of Weiner reported by Southcott & Kingston (1959). An investigation into the nature of the haemolytic activity will be reported elsewhere (Keen & Crone, unpublished). The haemolytic activity seems to be an integral part of the toxic activity, although the associated rise in plasma K^+ was not consistently high enough to be causally related either to death or to the cardiac irregularities. It is possible, on the present evidence, to propose that the toxin(s) alter membrane permeability. The respiratory arrest, cardiotoxicity and haemolysis would all derive from this permeability change, and may reflect the sensitivity of the target organs to such a change. Such a permeability change may be responsible for the pain and whealing experienced by people who are stung by *Chironex*. Potassium accumulation at nerve endings in the skin produces pain (Keele & Armstrong, 1964) ; changes in capillary permeability may be responsible for oedema.

It is of interest to compare *Chironex* toxin with the preparation from *Physalia* which was investigated by Lane (1967). That author noted cardiotoxic and haemolytic effects similar to those produced by *Chironex*. It is likely, however, that the *Chironex* toxin is either more potent or more abundant than in *Physalia*, for Southcott (1959) could find no evidence of fatalities following *Physalia* stings. The toxin also bears some resemblance to streptolysin O (Halpern & Rahman, 1968)

and the basic proteins of cobra venom (Wolff, Salabe, Ambrose & Larsen, 1967). Such resemblances confirm the hypothesis that *Chironex* toxin acts by causing a membrane permeability change but offer no indication of the chemical nature of the toxic material.

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(*Received November* 18, 1968)

IONIC INTERACTIONS IN ACETYLCHOLINE CONTRACTION OF THE DENERVATED RAT DIAPHRAGM

BY

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Reprinted from BRITISH JOURNAL OF PHARMACOLOGY, *July*, 1969, *vol.* 36, *No.* 3, *p.* 510.

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Br. J. Pharmac. (1969), **36**, 510–522.

Ionic interactions in acetylcholine contraction of the denervated rat diaphragm

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1. The nature of the drug-receptor interaction in the acetylcholine-induced contraction of the denervated rat diaphragm was studied both by altering the external ionic environment and by determining its drug sensitivity.

2. The response to acetylcholine was insensitive to tetrodotoxin or saxitoxin, but was abolished by procaine.

3. It was unaffected by levels of $MnCl_2$ sufficient to block the response of the innervated diaphragm to electrical stimulation, although higher levels reduced the response. The effect of Mn^{++} on the innervated diaphragm was overcome by raising the external Ca^{++} level; this was ineffective in the denervated preparation.

4. In spite of its insensitivity to tetrodotoxin the acetylcholine contraction was reduced and prolonged by low external Na^+ levels. This prolongation was not found when Li^+ substituted for Na^+.

5. Increasing the external level of Ca^{++} or Mg^{++} 3 to 5-fold reduced the acetylcholine contraction; high Ca^{++} also prolonged it. Reduction in the divalent cation level was without effect.

6. Procaine inhibition of the acetylcholine response was largely competitive, as was inhibition due to (+)-tubocurarine. This was shown by probit analysis and the dose-ratio test.

7. Thiocyanate (12 mM) augmented and prolonged the contraction; this action was modified by altering the Ca^{++} or Mg^{++} level of the solution.

8. The acetylcholine receptor resembles that of the innervated postsynaptic membrane.

The pharmacology of the acetylcholine-induced contraction of denervated muscle is of interest both for the observation of denervation phenomena and because the nicotinic receptor may be conveniently studied in such a preparation. Axelsson & Thesleff (1959) noted that denervated muscle responded to drugs in a manner qualitatively similar to the innervated endplate. Beránek & Vyskočil (1967) reported, however, that the acetylcholine (ACh) receptor of denervated muscle is less sensitive to (+)-tubocurarine (TC) blockade than is the innervated endplate, and Loomis & Konker (1967) reported that TC produces a contracture of the intact

denervated anterior tibial muscle of the rat. This finding confirmed the earlier report of McIntyre & King (1943).

It is of interest, therefore, to study the ACh receptor of the denervated rat diaphragm in an attempt to extend the observations of del Castillo & Katz (1954, 1955) on the post-synaptic ACh receptor, and to determine wherein the denervated preparation differs from it. To this end the effects of changes in the ionic environment on the ACh-induced contraction and interactions between ACh and a number of anticholinergic drugs have been studied.

Methods

Male hooded rats of the Wistar strain were anaesthetized with pentobarbital 40–50 mg/kg and the left diaphragm was denervated by evulsion of the phrenic nerve in the neck. They were maintained postoperatively for 8–21 days, to allow the nerve to degenerate. The ACh-induced contraction was constant during this period, as noted by Elmqvist & Thesleff (1960). The animals were then killed by a blow on the head, exsanguinated, and the innervated and denervated diaphragms set up as described previously (Freeman, 1968). Isometric tension developed by the muscles in response to drugs or electrical stimulation was recorded with Statham UC2 transducing cells and a Beckman type R dynograph recorder. Innervated preparations were stimulated either directly or through the nerve with 0·1 msec rectangular pulses of supramaximal voltage.

The standard nutrient solution used has been described elsewhere (Freeman, 1968). Na^+-free solutions were buffered with tris(hydroxymethyl)aminomethane hydrochloride (Tris). They were aerated with oxygen rather than the 95% oxygen and 5% carbon dioxide mixture used in the standard solution. Drugs were injected into the stream of gas bubbles which was used to aerate and to stir the organ baths. The bath temperature was monitored continuously with a thermistor. Unless otherwise stated experiments were carried out at 29° ±0·2° C.

The thickness of the muscles was determined at the end of experiments from their weight and surface area, assuming a density of 1·05. The mean thickness was 0·63 mm, and the range was from 0·58 to 0·74 mm. This agrees with our previous estimate of 0·66 mm (Freeman, 1968) and indicates that at the time of measurement denervation had not altered the thickness of the muscles. The contractile response to ACh or carbamylcholine was constant over a 5 hr experimental period, and tachyphylaxis was not evident provided each drug addition to the organ bath was separated by a 10 min wash period. Typical dose-response curves are shown in Fig. 2. The slope varied slightly from one preparation to another, consequently whenever possible each preparation was used as its own control.

TABLE 1. *Time course of acetylcholine or carbamylcholine contraction of denervated diaphragm*

	Rise time (sec)	Time to half relaxation (sec)	Maximum tension (g)
ACh $5{\cdot}5\times10^{-6}$M	12·0±0·4 (45)	46±2 (45)	5·5±0·3 (45)
Carbachol $7{\cdot}3\times10^{-5}$M	45±4 (18)	52±3 (18)	7·9±0·4 (18)

Figures shown are ± S.E. of the mean. The number of observations is shown in parenthesis.

Results

Time course of the contractile response

Acetylcholine or carbamylcholine (carbachol) produced a dose-dependent increase in isometric tension in the denervated diaphragm. The dose-response curves for the two drugs were parallel, but 7–9 times more carbachol was needed to produce the same tension as ACh. The response to carbachol was, however, more prolonged. As can be seen from Table 1 this resulted more from a slower rate of rise of the carbachol contraction than from a prolongation of the time to half relaxation. The tension developed in response to either drug declined as an exponential function of time.

The time course of the ACh contraction was relatively independent of the concentration of ACh used. The rise time increased slightly over the range $2{\cdot}75 \times 10^{-6}$M to $2{\cdot}75 \times 10^{-5}$M, but the time to half relaxation fell with increasing ACh concentration from 64 sec at $2{\cdot}75 \times 10^{-6}$M to 31 sec at $2{\cdot}75 \times 10^{-5}$M.

The reason for the brevity of the ACh response is obscure. Lüllmann & Reis (1967) noted that the depolarization of the denervated diaphragm due to ACh persisted after the muscle had relaxed. Consequently it cannot be ascribed to membrane "desensitization," but is more likely related to a resequestration of reticular Ca^{++}.

The duration of the contraction must also be viewed in relation to the rate of diffusion of ACh through the preparation. Krnjević & Mitchell (1960) noted that the time of half clearance of ACh from the diaphragm was 1·5 min. Their preparations appear to have been 50% thicker than ours, but it is obvious that the ACh contraction is too brief for ACh to have diffused uniformly through the muscle. One must assume that the measured contraction is a sum of contractions and relaxations which occur as ACh diffuses through the preparation. Because of this, conditions which prolong or shorten the contractile event will cause an apparent increase or decrease in the peak tension developed. This effect may partly explain the finding of Letley (1960), which was confirmed by us, that the ACh contraction develops greater tension at 22° C than at 37° C. We observed that low temperature markedly prolonged the contraction.

Effect of alteration of the external ionic environment

Castillo & Katz (1955) and Jenkinson & Nicholls (1961) noted that ACh was able to decrease the membrane resistance of the endplate and denervated muscle respectively in preparations soaked in Na^+-free solutions. Castillo & Katz (1955) described this effect of ACh "as a short-circuit placed across a rectifying membrane," and concluded that the action of ACh is independent of the process of electrical excitation. If this is so the decrease in membrane resistance in Na^+-free solution will be associated with currents due to ions other than Na^+.

Sodium substitution with sucrose

The denervated diaphragm offers a convenient preparation in which to study the effect of Na^+ depletion on the response to ACh. Sucrose, lithium or Tris hydrochloride were used to maintain the osmotic pressure of the nutrient solution.

The replacement of half the external Na^+ with an osmotically equivalent amount of sucrose was without effect on the tension developed in response to ACh. Replacement of all but 22 mM Na^+ with sucrose resulted in a slow decline in the tension developed to a constant dose of ACh. The results varied slightly in the eight diaphragms tested, but in general the tension developed in response to ACh declined to between 25–50% of the control value over a period of 60–150 min. The rise time was unchanged, but there was a gradual increase in the time to half relaxation, until at 60–70 min the ACh response had become a persistent contracture. These findings are illustrated in Fig. 1. It was noteworthy that in one experiment there was a gradual recovery of tension between 60 and 100 min, until the response was of nearly normal force.

Replacement of all the external Na^+ with sucrose, using Tris as a buffering agent, accelerated the changes seen in 22 mM Na^+ (Fig. 1). In six experiments the ACh response started to decline after 10 min, and levelled off at approximately 25% of the control value at 30–40 min. It was still possible to obtain an ACh contraction after 120 min in Na^+-free solution, and again one preparation showed a partial restitution of contractile force after 60 min exposure. The rate of rise fell off somewhat after 70 min in Na^+-free solution, and the duration of the response increased until it equalled the time of exposure of the preparation to ACh. Washing the preparation with normal solution for 10–12 min returned the duration of contraction to less than normal; further, the tension developed was from 140–170% of the control value. These parameters returned to control values over a period of 30–40 min.

It is possible that the partial restoration of tension which was occasionally seen after prolonged exposure to low Na^+ or Na^+-free solution may be related to the partial dependence of the ACh contraction on the Na^+ gradient across the membrane. The initial loss of extracellular Na^+ would reduce this gradient, which would tend to be restored by a later loss of intracellular Na^+ (Simon, Shaw, Bennett & Muller, 1957). Some evidence for this hypothesis is derived from the observation that the restitution of extracellular Na^+ brought about an initial increase in the ACh response to

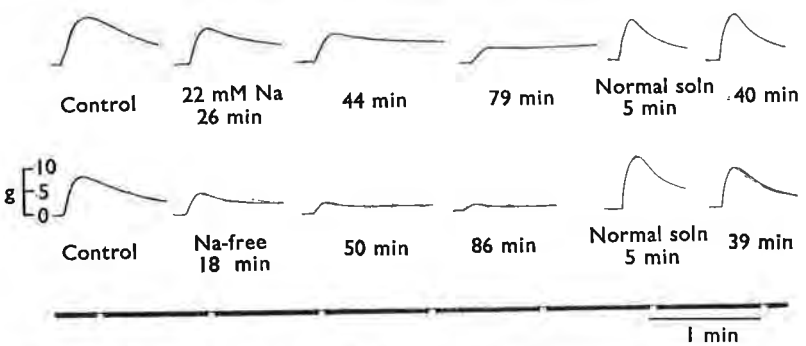

FIG. 1. Effect of Na^+ depletion on the ACh contraction of denervated diaphragm. The top row shows a control contraction (ACh 10^{-5}M), followed by contractions elicited in the presence of 22 mM Na^+, sucrose solution. The second row shows a control contraction by the same ACh concentration, followed by contractions elicited in the presence of Na^+-free sucrose solution. Return to the normal solution temporarily potentiates the contractile force above the control level. In each instance ACh was washed out at the conclusion of the tracing.

greater than normal levels ; one would expect the Na^+ gradient to be temporarily increased.

Exposure of a preparation to a Na^+-free sucrose solution which was also Ca^{++}-free resulted in a marked slowing of the rate of rise of the contraction and a reduction in tension over a period of 20 min. By 40 min the ACh response was abolished. The ineffectiveness of ACh in solutions lacking both Na^+ and Ca^{++} recalls the similar findings of Pappano & Volle (1966), who used the perfused superior cervical ganglion of the cat.

As would be expected, transmission was blocked by Na^+-free solutions in contralateral, innervated preparations in 10–15 min.

Sodium substitution with Tris

Experiments were carried out in which an attempt was made to maintain the ionic strength of the bathing solution by substituting all the Na^+ with Tris hydrochloride. It was found that the response to ACh declined to 20–40% of the control value over a period of 20 min (four experiments). Rise time and duration were also shortened. These effects may have been partly due to Ca^{++} chelation by Tris (Mahler, 1961), so the Ca^{++} concentration of the solution was then raised to 4·5 mM ($\times 3$ normal). Tension developed was but slightly improved by the Ca^{++} increase, but the response lengthened until it equalled the time of exposure of the preparation to ACh.

Sodium substitution with lithium

Since Li^+ can replace Na^+ in maintaining the action potential of nerve and muscle (Gallego & Lorente de Nó, 1951), experiments were carried out in which all the external Na^+ was substituted with Li^+. Tris hydrochloride was used as buffer.

The rate of rise and relaxation time were not altered by Li^+ solution. However, tension development fell off slowly over a 60 min period until it was 30–40% of the control value. Restoring the normal Na^+ solution brought back tension development to the control level in 10 min. The response to ACh in Li^+ solution was further depressed by increasing the external Ca^{++} level to 4·5 mM ; in contrast to the findings in Tris and sucrose solutions, Ca^{++} increase did not prolong the contraction.

Additional experiments were carried out with Li^+ solutions without Ca^{++}. Here it was found that the ACh response declined to zero in 50 min. Restoring the Ca^{++} to normal (1·5 mM) at this point restored the ACh response to 60% of the control value.

Neuromuscular transmission in the contralateral innervated preparation was blocked completely in 6–8 min by Li^+ solution (Onadera & Yamakawa, 1966 ; Freeman, 1968). If the response of the postsynaptic membrane is reduced by Li^+ in a quantitatively similar way to that of the ACh receptor of denervated muscle, then the greater efficacy of Li^+ in blocking transmission must be related to a reduction in the presynaptic output of ACh.

Divalent cation changes

Reduction of the external Ca^{++} or Mg^{++} concentration to 0·15 mM and 0·1 mM respectively ($\times 0{\cdot}1$ normal) was without significant effect on the ACh-induced con-

traction. An increase in the Mg^{++} concentration to 5 mM for 10 min before administration of ACh reduced the tension developed to 50–60% of the control value, but did not alter the time course of the contraction. A threefold increase in Ca^{++} reduced the response to approximately 70% of the control value, and increased the relaxation time five-fold (see Fig. 3). A further increase in Ca^{++} to 7·5 mM converted the ACh response to a sustained contracture.

The decreased tension developed in high Ca^{++} or Mg^{++} solutions may reflect the membrane stabilizing effect of these ions, and recalls the results of Takeuchi (1963), who determined the effects of Ca^{++} and Mg^{++} on the ACh-induced conductance change of the endplate membrane. The prolongation of the contraction by raised Ca^{++} may be a function of the ability of the sarcoplasmic reticulum to re-sequester Ca^{++} in the face of continuing depolarization and an increased Ca^{++} gradient.

Effect of $MnCl_2$ on ACh response

It has been shown that the action potentials of crustacean muscles are due to an increase in conductance for divalent cations rather than for Na^+ (Hagiwara & Nakajima, 1966); it is likely that the rising phase of the action potential in vertebrate smooth muscle is also unrelated to an increased Na^+ conductance (Kuriyama, Osa & Toida, 1966). It appeared possible, therefore, that an increase in Ca^{++} conductance could be of major importance in ACh depolarization of denervated muscle. Manganese ions have been shown to suppress such "Ca^{++} spikes" (for references, see Hashimoto & Holman, 1967); consequently their effect on the ACh-induced contraction was determined.

It was found that 1 mM $MnCl_2$ added to a Tris-buffered Na^+ containing solution completely blocked the innervated diaphragm to both direct and indirect stimulation in 6 min. It was possible to break through the muscle membrane blockade by increasing the stimulating voltage from a normal value of 6–8 V for supramaximal stimulation to approximately 100 V.

This level of $MnCl_2$ was completely without effect on the ACh response of the denervated diaphragm, even after 30 min exposure to Mn^{++}. Increasing the Mn^{++} concentration to 4 mM reversibly depressed the ACh response. The dose-response curve in the presence of 4 mM Mn^{++} was parallel to the control, and the dose ratio was approximately 2.

Increasing the external Ca^{++} level to 4·5 mM completely relieved the blockade of the innervated preparation; all parameters returned to normal. However, a similar increase in Ca^{++} concentration did not affect the partial inhibition of the ACh response in the denervated preparation. Further, the effect of Mn^{++} on this response was not altered by concurrently reducing the Ca^{++} level to 0·15 mM, although the time to half relaxation was somewhat shortened in the low Ca^{++} solution.

Thus Mn^{++} appears to block the innervated preparation by processes involving competition with Ca^{++}; its inhibitory effect on the ACh response of the denervated preparation requires a higher concentration of Mn^{++} and cannot be modified by changes in the external Ca^{++} level.

Drug effects on ACh response

Tetrodotoxin and saxitoxin

Katz & Miledi (1966) noted that tetrodotoxin, which appears to inhibit specifically the inward Na^+ current during the action potential (Kao, 1966), was without effect on the electrical properties of the neuromuscular junction. This appears also to be so in the axo-axonic giant synapse of the stellate ganglion of the squid (Katz & Miledi, 1967). Saxitoxin is pharmacologically very similar to tetrodotoxin.

The effects of these drugs have been compared in both the innervated and denervated diaphragm preparations. It was found that tetrodotoxin at a concentration of 10^{-7} g/ml. completely blocked the innervated diaphragm to direct and indirect stimulation in 3 min ; approximately twice as much saxitoxin was required to block this preparation in the same time. These drug levels were totally without effect on the ACh contraction of the denervated diaphragm, even after exposure for 60 min. Additional experiments were carried out in which tetrodotoxin (10^{-7} g/ml.) was added to solutions in which all the Na^+ had been replaced with sucrose, lithium or Tris. Here again the contraction was not modified by the presence of tetrodotoxin.

Procaine hydrochloride

In distinction to tetrodotoxin and saxitoxin, procaine hydrochloride (PrHCl) was found to block reversibly the ACh response of the denervated diaphragm ; dose-response curves for ACh were shifted to higher concentrations. Figure 2 shows the effect of two concentrations of PrHCl on the ACh dose-response curve. As the control curves differed somewhat in slope the data could not be pooled ; the results

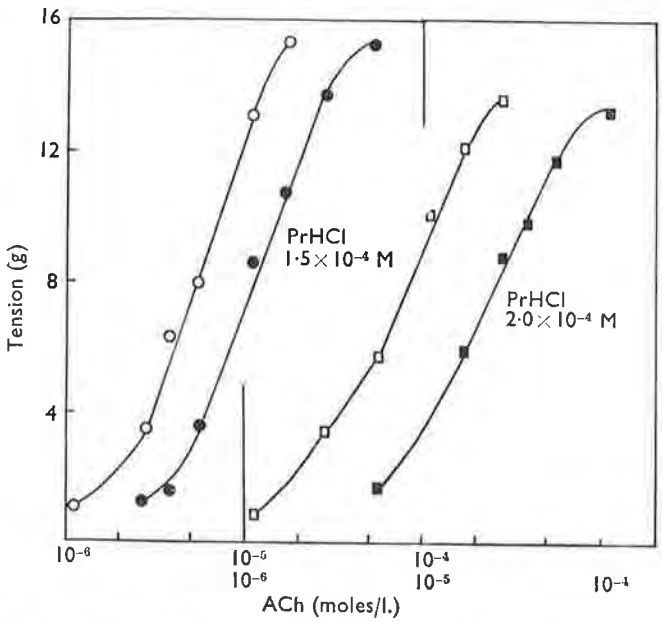

FIG. 2. Effect of procaine hydrochloride on the ACh dose-response curve. Two levels of PrHCl are shown. The graph has been divided to show two experiments ; the upper figures on the abscissa refer to the left hand segment, the lower to the right hand segment.

of two typical experiments are shown in Fig. 2. Using probit analysis the difference in slope in each case was shown not to be significant at the 5% probability level. Further it was possible to equal the maximum ACh contraction in the presence of PrHCl by raising the ACh level. Higher concentrations of PrHCl were tested in order to extend the data ; it was found, however, that at 3 or 4×10^{-4}M it was impossible to obtain a steady response to ACh. The data illustrated in Fig. 2 were obtained by adding ACh to the organ bath after 10 min exposure to PrHCl ; the preparation was then washed for 5 min and PrHCl added for 10 min before the next ACh dose. This procedure did not produce consistent responses with higher levels of PrHCl and one must assume that a sufficient quantity of the drug had entered the cell to interfere with Ca^{++} release by the reticulum (Bianchi, 1968).

In order to test the apparently competitive nature of the PrHCl inhibition over the limited concentration range available, we carried out the dose-ratio test described by Paton & Rang (1965). Tubocurarine was used as the second ACh antagonist. The finding of Elmqvist & Thesleff (1960) that TC competitively inhibits the ACh response of the denervated diaphragm was confirmed. Dose response-curves were drawn, and were found to be parallel over the range of concentrations of TC shown in Table 2.

The dose ratio (DR) is defined as D_1/D where D_1 is the dose of an agonist in the presence of an inhibitor, required to produce the same response as the concentration of the agonist, D, in the absence of inhibitor. If two antagonists giving dose ratios DR_1 and DR_2 are both competitive inhibitors of the agonist (ACh) the dose ratio obtained in the presence of both inhibitors (DR_{1+2}) should equal $DR_1 + DR_2 - 1$. If the two inhibitors do not compete for the same active site on the receptor $DR_{1+2} = DR_1.DR_2$.

Experiments to test the nature of PrHCl inhibition were carried out in the following manner. First, the ACh dose-response curve for each preparation was determined. The dose ratios for TC and PrHCl were then determined separately, and then the combined dose ratio DR_{1+2} was determined. A difficulty was encountered in that the combined dose of TC and PrHCl caused a prolonged inhibition of the ACh response which persisted for upwards of an hour in some preparations. Consequently it was found difficult to obtain more than one or two values of DR_{1+2} in each preparation. It was found (see Table 2) that the values obtained were consistent with competitive inhibition, or were slightly too large. In no case was $DR_{1+2} = DR_1.DR_2$. Thus one may conclude that PrHCl is essentially a competitive inhibitor of ACh, but that its action also contains a non-competitive element.

Interactions between Ca^{++}, Mg^{++}, PrHCl and ACh were also studied, in an attempt to separate the membrane effects of PrHCl from those operative at the reticular level (Bianchi, 1968). Thus Ca^{++} and Mg^{++} may be expected to have similar effects on the membrane (Takeuchi, 1963) but Mg^{++} cannot substitute for

TABLE 2. *Dose ratio test for ACh antagonism by TC and procaine*

TC (M)	Procaine (M)	DR_{1+2}	$DR_1 + DR_2 - 1$	$DR_1.DR_2$
10^{-7}	$1{\cdot}5 \times 10^{-4}$	2·9	3·2	4·4
10^{-7}	$1{\cdot}5 \times 10^{-4}$	4·4	4·0	6·2
2×10^{-7}	$1{\cdot}5 \times 10^{-4}$	6·7	4·8	7·9
3×10^{-7}	$1{\cdot}5 \times 10^{-4}$	18·5	11·0	32·0
4×10^{-7}	$1{\cdot}5 \times 10^{-4}$	5·0	6·4	8·9

Ca^{++} in excitation-contraction (E-C) coupling. The results of typical experiments are shown in Fig. 3. As was noted earlier, low Ca^{++} or Mg^{++} solutions did not affect the ACh response. The inhibitory response to PrHCl ($1{\cdot}5 \times 10^{-4}$M) was diminished by concurrently lowering either the Ca^{++} or Mg^{++} concentration. This effect was particularly marked in 0·15 mM Ca^{++}. Increasing the level of divalent cation increased the inhibitory power of PrHCl. Mg^{++} (5 mM) did not affect the time course of the contraction; 4·5 mM Ca^{++} both reduced the amplitude and prolonged the ACh contraction. Thus both PrHCl and divalent cations appear to stabilize the membrane and their effects are synergistic.

PrHCl was found to block neuromuscular transmission in contralateral innervated preparations at approximately 5×10^{-4}M. It was noteworthy that the blockade could be relieved by increasing the Ca^{++} level of the solution to 4·5 mM. Relief was never complete; twitch tension was restored to 50–70% of the control level. Presumably the reduction in amplitude of the nerve action potential by procaine inhibited ACh release. This effect was overcome by raising the Ca^{++} level. Post-synaptic inhibition by procaine may well have been potentiated by raised Ca^{++}.

Effect of thiocyanate on ACh response

If one assumes that PrHCl acts at the level of the ACh receptor to block the initial excitatory event in the E-C coupling sequence then it is of interest to determine interactions between ACh and events occurring more distally in the E-C sequence. SCN^- is the most active of a lyotropic series of anions which in innervated amphibian muscle are believed to act on the transverse tubular element, to alter the E-C process so as to increase the duration of the active state (Bianchi, 1968; Hodgkin & Horowicz, 1960).

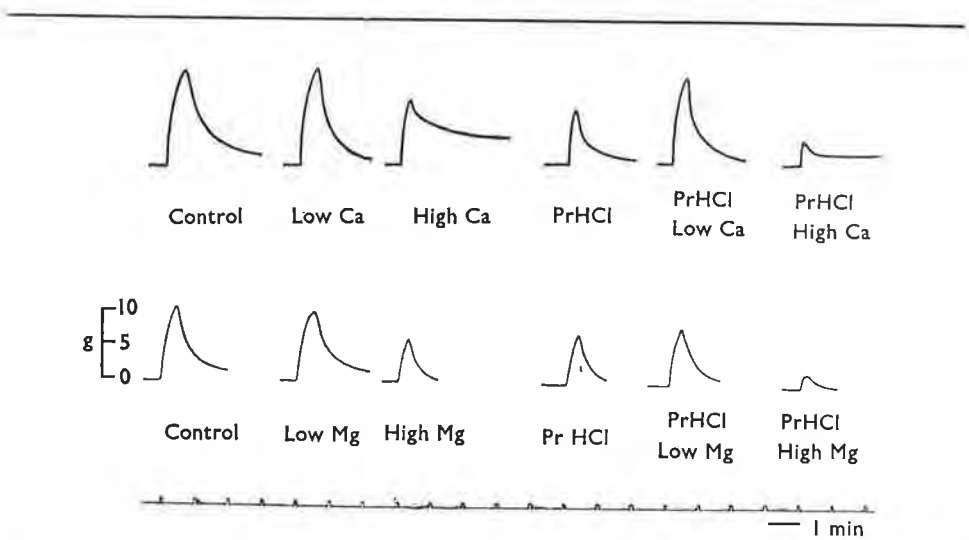

FIG. 3. Effect of divalent cation variation on the inhibition by PrHCl of the ACh response. The upper row shows a control contraction (ACh 10^{-5}M), followed by contractions in the presence of 0·15 mM Ca^{++}, 4·5 mM Ca^{++}, $1{\cdot}5 \times 10^{-4}$M PrHCl, PrHCl + 0·15 mM Ca^{++}, and PrHCl + 4·5 mM Ca^{++}. The lower row shows a control contraction followed by contractions in the presence of 0·1 mM Mg^{++}, 5 mM Mg^{++}, $1{\cdot}5 \times 10^{-4}$M PrHCl, PrHCl + 0·1 mM Mg^{++}, and PrHCl + 5 mM Mg^{++}. In each instance the drugs were washed out at the end of each tracing.

Experiments were carried out to determine the effect of replacing 12 mM Cl^- in the bathing solution with SCN^-. The ACh response was both potentiated and prolonged. The effect of SCN^- on the ACh dose-response curve is illustrated in Fig. 4. It may be noted that the curves diverged at high levels of ACh, suggesting that the prolongation of the contraction was sufficient to augment the contraction height

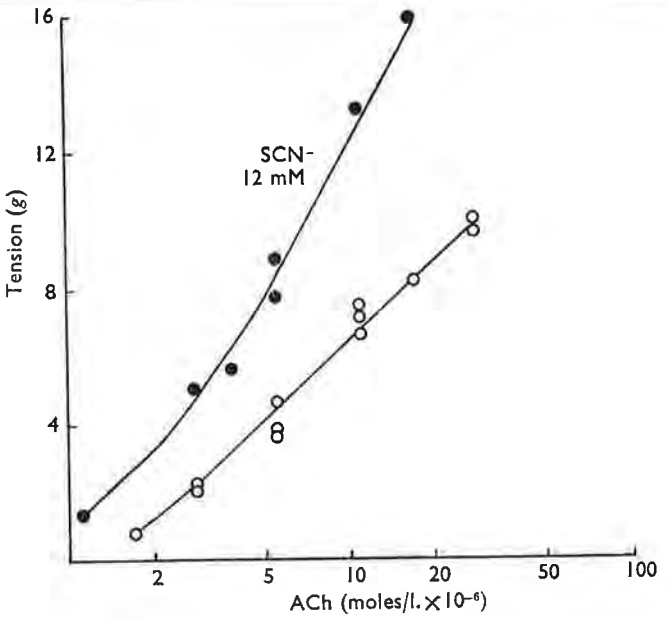

FIG. 4. Effect of 12 mM SCN^- on the ACh dose-response curve.

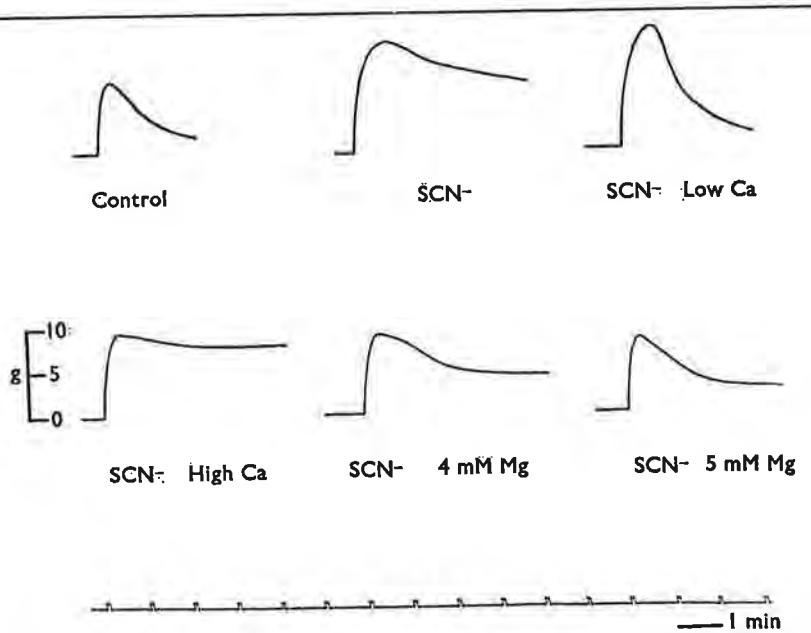

FIG. 5. Effect of divalent cation variation on the SCN^- potentiation of the ACh response. The upper row shows a control response (ACh 10^{-5}M), followed by the potentiated response in 12 mM SCN^-. Low Ca^{++} (0·15 mM) increases the amplitude, 4·5 mM Ca^{++} (lower row) reduces and prolongs the response. High Mg^{++} (4 or 5 mM) reduces the response.

(see above). Both the amplitude and duration of the ACh response in the presence of SCN^- were dependent on the Ca^{++} and Mg^{++} concentration of the external medium. Figure 5 shows that reduction of the external Ca^{++} level to 0·15 mM reduced the duration of the contraction, and slightly increased the amplitude. Increasing the external Ca^{++} level to 4·5 mM reduced the amplitude of the ACh contraction and prolonged it until it equalled the time of exposure to ACh. On the other hand, increasing the external Mg level to 4 or 5 mM reduced the amplitude to that of the control without SCN^-. The contraction was still, however, prolonged by a factor of two compared to the control contraction. Thus the ability of SCN^- to augment and prolong the ACh response can be modified by Ca^{++} and Mg^{++}.

It may be noted that SCN^- augmented but did not prolong the indirectly elicited twitch of the contralateral innervated preparation.

Discussion

The response of the chronically denervated diaphragm to acetylcholine appears in general to resemble that of the postsynaptic membrane. Evidence from studies involving alteration of the external ionic environment confirms the thesis of del Castillo & Katz (1955) that ACh " short-circuits " the excitable membrane, allowing ionic currents to flow according to their gradients. Thus the ACh contraction can be related to the Na^+ gradient across the membrane, although it is not entirely dependent upon the presence of Na^+. It may be that under conditions of almost total Na^+ depletion movement of Ca^{++} is sufficient to initiate contraction. It is of interest that the lengthening of the ACh contraction seen in low Na^+-sucrose solutions did not occur when Tris or Li^+ was substituted for Na^+. Raising the Ca^{++} concentration in Tris solution, as in Na^+ solution, prolonged the response. This prolongation was not seen in Li^+ solution when external Ca^{++} was raised. Thus Li^+ appears to be more effective than Na^+ in maintaining the relaxing mechanism.

The reason for the brevity of the ACh response is obscure. It cannot be related to ACh hydrolysis, because the denervated preparation contains AChE only in the region of the degenerating synapse (Eränkö & Teräväinen 1967). Further, the carbachol response, although longer than the ACh response, is nevertheless of finite length. The observation of Lüllmann & Reis (1967) that the denervated diaphragm relaxes in spite of a continuing depolarization suggests that, as in a K^+-induced contraction, the sarcoplasmic reticulum is able to re-sequester Ca^{++} although the membrane remains depolarized. This observation makes a comparison of the duration of conductance changes at the endplate with the contractile event hazardous.

The involvement of the reticular relaxing system in the ACh contraction means that drug interactions may occur at three possible points: first at the ACh receptor which may be located on the surface membrane, second at the level of the transverse tubule where the membrane event is propagated into the muscle cell, and third at the level of the terminal cisternae, where Ca^{++} is released to initiate contraction.

There is some evidence (Brody, 1966 ; Howell, Fairhurst & Jenden, 1966) that the reticular relaxing system alters following denervation, and has an increased ability to sequester Ca^{++}. Brody (1966) suggested that E-C coupling is facilitated after denervation, and that this may be a factor in producing fibrillations.

The finding that the ACh response is totally unaffected by concentrations of tetrodotoxin or saxitoxin that rapidly block the innervated preparation confirms

the thesis that the permeability changes following ACh-receptor interaction differ from those involved in the generation of the action potential. Whether the tetrodotoxin-sensitive receptor and the ACh receptor exist in series or in parallel is less clear. It is possible that the ACh receptor may develop in response to denervation in the transverse tubular element, and thus occur more distally in the chain of E-C coupling than the tetrodotoxin receptor.

The findings that the ACh receptor is less sensitive to Mn^{++} than the innervated muscle rules out the possibility that the ACh generates a "Ca^{++} spike" such as occurs in smooth and crustacean muscle. The insensitivity of the Mn^{++} inhibition of the ACh receptor to variation in external Ca^{++} suggests that Mn^{++} is not competing with Ca^{++}, but offers no indication as to its site of action.

If it be granted that SCN^- affects the E-C coupling sequence by lowering the threshold for excitation at the level of the transverse tubule system, then the ACh receptor must be located either at this level or more proximal to it. The potentiation of the ACh response by SCN^- was reduced by increased concentrations of either Ca^{++} or Mg^{++}. This may represent stabilization of the ACh receptor, as has been suggested for PrHCl.

It is of interest that Ca^{++}, Mg^{++} and PrHCl synergize in denervated muscle. Other workers have noted antagonism between PrHCl and Ca^{++} in other systems. Thus in smooth muscle (Feinstein, 1966), lobster nerve (Blaustein and Goldman, 1966) or frog spinal ganglia (Aceves and Machne, 1963) Ca^{++} increase relieved local anaesthetic inhibition. These authors attributed their results to competition between PrHCl and Ca^{++} at a membrane site which controls the increase in Na^+ conductance upon electrical stimulation. Our finding again emphasizes the separateness of the membrane permeability changes following ACh, and the changes associated with electric excitation.

We wish to thank Dr. E. J. Schantz, Fort Detrick, Maryland, for a gift of saxitoxin.

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(*Received January* 20, 1969)

Toxicon, 1969, Vol. 7, pp. 277–286. Pergamon Press. Printed in Great Britain

EFFECTS OF *CHIRONEX FLECKERI* TOXIN ON THE ISOLATED PERFUSED GUINEA PIG HEART

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(*Accepted for publication* 16 *August* 1969)

Abstract—The effects of toxin extracts from the cnidarian, *Chironex fleckeri* Southcott have been studied on the isolated, perfused guinea pig heart. Small doses of toxin resulted in reversible decreases in coronary flow, heart rate and amplitude of contraction whilst larger doses produced irreversible changes. Although the toxin did not appear to act at alpha or beta adrenergic receptors, catecholamines provided some protection. Serotonin was devoid of any protective action. Adenosine or ATP did not affect the changes in coronary flow or heart rate caused by the toxin, but reduced the amplitude changes.

It is suggested that the different effects of the vasodilator agents on the toxin response are due to their differing sites of action within the coronary circulation. Stimulation of cardiac metabolism by the catecholamines and adenosine or ATP may also be a factor in the maintenance of cardiac contractility by these compounds in the presence of the toxin. The effects of the toxin on the isolated heart are correlated with those observed in previous experiments on the intact animal.

INTRODUCTION

STINGINGS due to the box jellyfish, *Chironex fleckeri* Southcott have caused a number of deaths in Northern Australian waters (BARNES, 1966, 1967).The abundance of the animals during the summer months results in a public health hazard which warrants a study of the pharmacological properties of the toxin in some detail.

It was shown previously (FREEMAN and TURNER, 1969) that death in experimental animals is characterized by respiratory arrest with concurrent signs of marked cardiotoxic activity. The heart was slowed, irregular and showed varying degrees of conduction delay terminating in atrioventricular block. A decrease in cardiac output coincided with the development of bradycardia. ECG changes were consistent with an interference in both atrioventricular conduction and repolarization of the cardiac muscle.

The present study has been concerned with the effects of the toxin on the isolated perfused guinea pig heart, in an attempt to characterize the cardiotoxin further.

METHODS

Hearts from guinea pigs weighing between 300 and 450 g were used throughout this study. Animals were anaesthetized with a 50 per cent CO_2: 50 per cent O_2 mixture (SATCHELL *et al.*, 1968) or ether; the heart was rapidly removed, the aorta cannulated and the preparation mounted in the perfusion apparatus (SATCHELL, 1967). The time from opening of the thoracic cavity to the beginning of perfusion was 3–4 min. Twin reservoir systems enabled rapid changes of fluid composition when required. The perfusion pressure was 65 cm water.

Heart rate and isotonic contraction were measured with a differential transformer. This consisted of three 120 Ω coils with a 3 V 400 Hz signal supplied to the centre coil. Vertical movement of a ferrite core centrally produced an output across the outside coils. For use, the coils were mounted in a perspex block surrounding a tubular extension at the base of the heart chamber. A weighted ferrite core (3·2 g) was attached to the apex of the heart, the vertical movement of this core providing a measure of myocardial contraction. The response of this device was linear over the range used and a pen deflection of 6·3 mm was equivalent to 1 mm movement of the core. Isotonic contraction has been used by various workers (Lu and Melville, 1951; Zachariah 1961; Nayler and Emery, 1962) as a measure of ventricular function. The heart chamber and transducer are illustrated diagrammatically in Fig. 1.

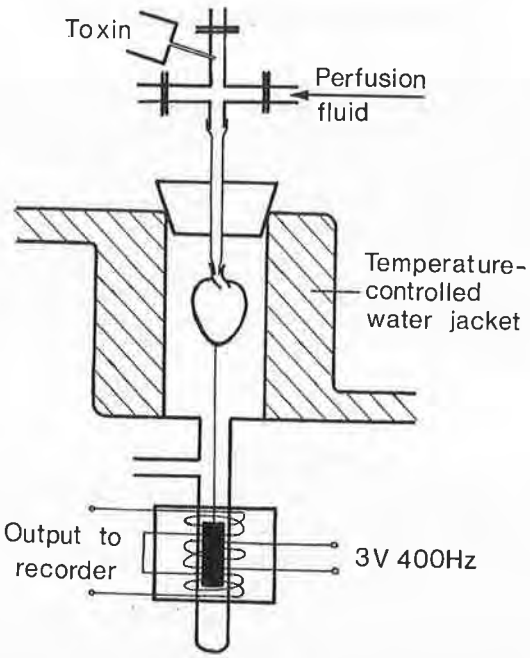

Fig. 1. Diagram of heart chamber and differential transformer transducer.
The three-way connector above the chamber allows entry of perfusion fluid from either of two sources; the third arm is for removal of gas bubbles. The differential transformer could be moved in a vertical plane to enable alignment with the ferrite core.

Perfusion fluid enters the coronary circulation from the aorta and passes via the coronary veins to the right side of the heart from which it empties. Coronary flow was calculated by conversion of the number of drops per min measured by a drop counter placed at the outlet from the heart chamber to ml per min.

Outputs from the differential transformer and the drop counter were recorded on a Beckman type RP dynograph recorder.

All preparations were perfused for a 20 min equilibration period and experiments were then conducted over the following 90 min. After the initial equilibration period the parameters measured remained constant for approximately two hours. The recording showed no appreciable base line shift representing overall contraction or relaxation of the heart over this period.

The normal perfusate had the following composition: NaCl 115mM, KCl 4·6mM, $CaCl_2$ 1·8mM, $MgSO_4$ 1·2mM, NaH_2PO_4 1·2mM, $NaHCO_3$ 22mM, glucose 22mM. The solution was bubbled with 95 per cent O_2:5 per cent CO_2 and was filtered through a sintered glass filter in the perfusion reservoir (BLEEHEN and FISHER, 1954). In some experiments 1 per cent dextran (Macrodex, Pharmacia) was added to the bathing solution as a possible method of reducing oedema in the heart. However, it appeared to have little or no effect and so was omitted in later experiments. Ascorbic acid (0·1mM) was added to all solutions for the experiments involving catecholamines (SATCHELL *et al.*, 1968). This level of ascorbic acid had no observable effect on the parameters measured. The temperature of the perfusate was 37·0±0·3°.

For most of this work the toxin was prepared by suspension of portions of frozen tentacle in 154 mM NaCl and homogenization for 1 min using a mechanical tissue disintegrator (Ultra-Turrax, Janke and Kunkel Kg.) followed by centrifugation at 5°. The resulting supernatant was divided into 0·5 ml ampoules and stored at −15°. This enabled small amounts of toxin to be thawed and used at any one time; samples were used within 20 min of thawing and were not refrozen. For the dose–response data, separate samples were thawed and diluted for each level as required. The pharmacological properties of NaCl-extracted toxin, amnion 'milked' toxin (BARNES, 1967) and a preparation partially purified by Sephadex filtration (FREEMAN and TURNER, 1969) are identical. It was found in the present study that these three preparations had identical effects on the isolated perfused heart. This would be expected since the extremely high toxicity of the tentacle extracts (all contained more than 100 mouse units per mg tentacle) made extensive dilution necessary before use. Thus modification of the action of the toxin extracts by other tissue constituents would be unlikely. Further, FREEMAN and TURNER (1969) were unable to detect any difference in the pharmacological properties of the three toxin preparations using a wide range of pharmacological tests. The extract has been further purified without alteration in its properties (CRONE and KEEN, 1969).

The potency of the toxin was assayed by i.v. injection of 20 g mice, the minimum lethal dose determined in this way being a 'mouse unit' (FREEMAN and TURNER, 1969). The standard dose administered to the heart was 0·03 'mouse units'. The potency was rechecked several times during the course of the experiments to ensure that the dose of toxin remained constant.

The toxin sample was added to the perfusion fluid at a point just proximal to the heart. As there was no recirculation there was only one contact between the heart and the toxin; recirculation, however, would have had little effect as the toxin when dilute is extremely labile at 37°.

As the response to *Chironex* toxin, at the level used, was transient and therefore did not reach a steady state, modification of the toxin response by drugs could only be investigated by measurement of the changes in cardiac parameters produced by the toxin in the presence of a steady response to the particular drug being used. The dose levels of the catecholamines and other drugs employed throughout this study were therefore chosen in preliminary experiments as representing the minimum dose consistent with a steady response of reasonable magnitude. The doses of alpha and beta blocking drugs are minimum blocking doses.

In all experiments using vasodilators the changes in cardiac parameters are expressed as percentages. These were calculated using the control level over 2–3 min prior to addition of either the toxin or the vasodilator as 100 per cent. The percentage changes in flow, rate and amplitude due to *Chironex* toxin in the presence of a vasodilating agent are calculated using the steady state levels obtained in the presence of the vasodilator as 100 per cent.

Drugs used in the study were: L-ascorbic acid (May and Baker), L-epinephrine bitartrate (K and K), L-norepinephrine bitartrate (Levophed, Winthrop), isopropylnorepinephrine sulphate (isoproterenol) (Burroughs Wellcome), phentolamine methanesulphonate (CIBA), propranolol hydrochloride (ICI), serotonin creatinine sulphate (Koch-Light), adenosine (Koch-Light), di-sodium adenosine-5′-triphosphate (Sigma).

RESULTS

Effects of Chironex *toxin on the untreated heart*

The control values for coronary flow rate, heart rate and amplitude were consistent throughout the series of experiments. Coronary flow had a mean rate of 10·5±0·5 ml per min (± S.E. of 56 observations), and mean heart rate was 214±4 beats per min (± S.E. of 56 observations). These values are consistent with those of other workers (WESTFALL, 1968). The height of the recorded amplitude trace is proportional to the peak amplitude of the isotonic contraction. This parameter is convenient to measure and appears to be linearly related to other indices of ventricular function (OPIE, 1965). A mean value of 21·0±0·5 mm deflection (±S.E. of 56 observations) was obtained.

The injection of a standard dose of 0·03 'mouse units' of toxin into the coronary perfusion circuit brought about reversible changes in the three parameters being measured.

There was an immediate decrease in coronary flow rate which persisted over the first minute and was followed by a slower return to the control level over the next 4–7 min. The decrease in coronary flow was accompanied by a negative chronotropic response on a slower time scale. The heart rate decreased over the first 2–3 min and then returned to control levels within 10 min of administration of the toxin. A decrease in amplitude was also observed; this followed a similar time course to the rate change. The toxin produced no significant overall contraction or relaxation of the heart. These effects are illustrated in Fig. 2 which shows the response to three consecutive doses of *Chironex* toxin in one heart.

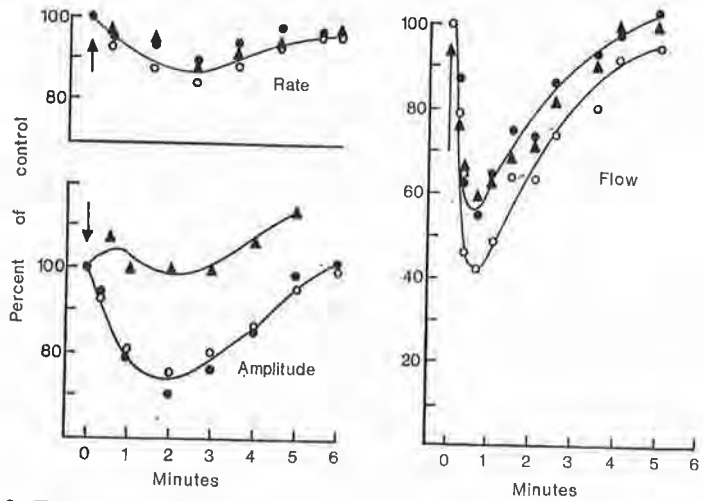

FIG. 2. EFFECT OF *Chironex* TOXIN ON HEART RATE, AMPLITUDE OF CONTRACTION AND CORONARY FLOW.

Three successive doses (each 0·03 'mouse units', added at arrow) separated by 15 min rest periods have been administered to one heart. The variation in amplitude response shown is an extreme case. ○——○ first dose; ●——● second dose; ▲——▲ third dose.

Injections of toxin were separated by periods of 10–15 min throughout the course of all experiments. Coronary flow and heart rate changes were reproducible both within experiments and between experiments, but amplitude changes were subject to some variation. In Fig. 2 the amplitude changes in response to the first and second doses of toxin are identical but the third dose of toxin has had little effect. In most experiments, however, the degree of variation was less than this.

The dependence of changes in coronary flow, heart rate and amplitude on the level of toxin was also investigated. These results are illustrated in Fig. 3. The percentage change

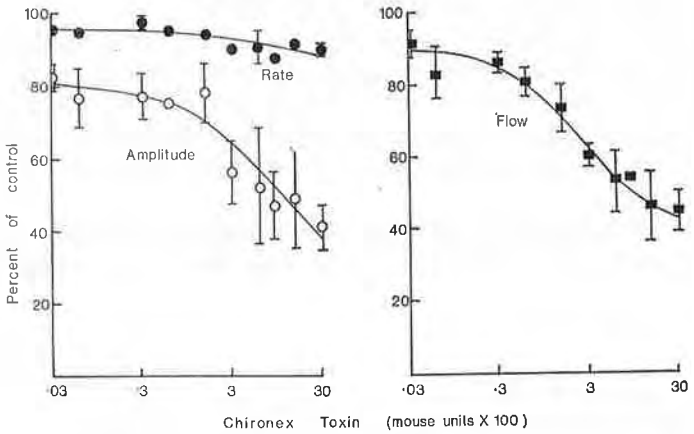

FIG. 3. EFFECT OF A RANGE OF DOSES OF TOXIN ON HEART RATE, AMPLITUDE OF CONTRACTION AND CORONARY FLOW.
Semilogarithmic plot; the error bars represent ± S.E. of mean of 4–9 observations. Where error bars are absent, the standard error was less than 1·5 per cent.

in coronary flow was calculated using the control level over 2–3 min prior to addition of toxin and the minimum rate of flow obtained within 1 min after the addition of the toxin. Rate and amplitude changes were calculated similarly except that the minimum rate and amplitude obtained within 3 min of the addition of the toxin was used.

The dose–response curve of change in coronary flow against log dose of toxin is sigmoid over the heart of concentrations employed.

The decrease in heart rate was relatively constant over the range of toxin concentrations employed.

Amplitude response to low levels of toxin was similar to that obtained with coronary flow but at higher levels there was no tendency for the curve to flatten; instead amplitude declined to zero. It can be seen from the standard error values in Fig. 3 that the change in amplitude is subject to increasing variation with increasing levels of toxin.

Doses of toxin up to 0·3 'mouse units' evoked reversible changes in coronary flow, heart rate and amplitude of contraction. At or above this dose, these changes became partially or completely irreversible. Heart rate became irregular at levels greater than 0·3 'mouse units' and at doses of 3·0 'mouse units' ventricular contraction was usually abolished although the atria continued beating for some time (FREEMAN and TURNER, 1969).

The marked ability of the toxin to reduce coronary flow prompted an investigation of the effects of several vasodilator agents on this response.

Effects of Chironex *toxin in the presence of catecholamines*

When epinephrine (0·3 μM), norepinephrine (0·3 μM) or isoproterenol (0·36 μM) was added to the perfusing fluid the following changes were observed. Coronary flow was increased, the most marked rise being produced by isoproterenol which increased flow to 160 per cent of the control level; epinephrine was less effective and norepinephrine was least effective. Heart rate was increased to the same extent by each compound and amplitude of contraction was increased equally by epinephrine and isoproterenol. Norepinephrine had little augmenting effect on amplitude and in some preparations a decrease in amplitude was observed. The effects of catecholamines are consistent with those obtained by other workers. (SATCHELL *et al.*, 1968; GLOMSTEIN *et al.*, 1967).

The effects of 0·3 μM epinephrine are illustrated in Fig. 4; epinephrine was added at zero time in each case. For each of the three compounds the maximum response was obtained after 2 min perfusion and remained constant over the next 10 min. This permitted the toxin to be added after 2 min and the course of its action to be followed in the presence of catecholamine. Control responses in the absence of catecholamines were obtained before and after this procedure.

The effect of *Chironex* toxin in the presence of epinephrine is illustrated in Fig. 4. The chronotropic response to the standard dose of toxin (0·03 'mouse units') under control

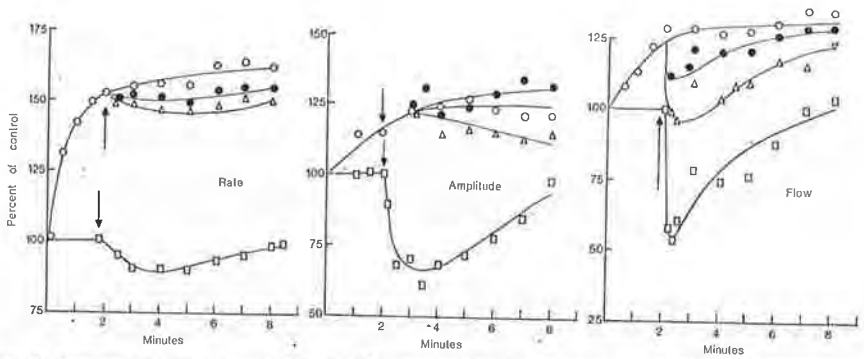

FIG. 4. EFFECT OF A STANDARD DOSE OF *Chironex* TOXIN IN THE PRESENCE OF 0·3 μM EPINEPHRINE. Epinephrine is added at zero time; toxin is added at the arrow. Percentage changes after addition of toxin in the presence of epinephrine are calculated using the heart rate, amplitude and coronary flow levels observed 2 min after addition of epinephrine as 100 per cent values. □——□ 0·03 'mouse units' toxin, normal perfusion fluid; ○——○ 0·3 μM epinephrine added to perfusion fluid at zero time; ●——● 0·03 'mouse units' toxin in presence of 0·3 μM epinephrine; △——△ 0·15 'mouse units' toxin in presence of 0·3 μM epinephrine.

conditions was small and it was not possible to say whether epinephrine had a significant effect on this change. However, the presence of epinephrine produced substantial effects on the changes in coronary flow and contraction amplitude resulting from toxin administration. The standard toxin dose administered in the presence of epinephrine produced no significant change in the amplitude and even five times the normal dose of toxin had little effect. The coronary flow in the presence of epinephrine was decreased only slightly by the normal toxin dose and five times this level (0·15 'mouse units') was required to approach the flow changes observed in the control.

Toxin administered in the presence of 0·36 μM isoproterenol produced similar effects. Decrease in coronary flow by the standard toxin dose was insignificant and even at five

times this dose the decrease was only about one-third of that observed in a standard control dose. Changes in rate and amplitude were similar to those observed in the presence of epinephrine. The standard toxin dose administered in the presence of norepinephrine produced similar results to those observed with epinephrine and isoproterenol.

Effect of Chironex *toxin in the presence of alpha or beta adrenergic blocking drugs*

As perfusion of catecholamines modified the response to the toxin, experiments were conducted to determine the effect of toxin on a heart in which either the alpha or the beta receptors were blocked by phentolamine or propranolol respectively.

Phentolamine (2·7 μM) and propranolol (3·9 and 7·8 μM) both decreased heart rate slightly and had little or no effect on coronary flow. Amplitude of contraction, however, showed a slow decline over the period of perfusion but returned to normal levels when normal fluid was restored.

The effect of the toxin on coronary flow, heart rate and amplitude or contraction was not significantly modified by the presence of phentolamine at the level used. Propranolol had no effect on amplitude or rate responses to the toxin, but the change in coronary flow was consistently reduced by a small amount when the toxin was injected in the presence of the beta blocking agent.

Effects of Chironex *toxin in the presence of serotonin*

The addition of 5 μM serotonin to the perfusion fluid produced increases in coronary flow, heart rate and amplitude of contraction. Coronary flow was increased by 34 per cent, heart rate was increased by 19 per cent and amplitude of contraction by 9 per cent (all increases are means of four observations). The responses reached a maximum within 2 min and then remained constant over the next 10 min.

Coronary flow and heart rate responses to *Chironex* toxin in the presence of serotonin were not significantly different from those obtained as controls in the absence of serotonin. The toxin appeared to have a greater effect on amplitude in the presence of serotonin than in the control situation but the difference was not great. The results are summarized in Table 1.

TABLE 1. CHANGES PRODUCED IN THE THREE HEART PARAMETERS AFTER 0·03 'MOUSE UNITS' *Chironex* TOXIN IN THE PRESENCE AND ABSENCE OF 5 μM SEROTONIN
Results are the mean of two experiments

	Heart rate (beats/min)	Amplitude of contraction (mm)	Coronary flow (ml/min)
Control	170	21	7·4
Control after toxin	150	17·5	4·0
5 μM Serotonin	200	22	9·9
Serotonin and toxin	180	17	5·3

Effects of Chironex *toxin in the presence of adenosine and adenosine triphosphate* (ATP)

Adenosine and ATP are both potent coronary vasodilators (WEDD, 1931; ROWE *et al.*, 1962; ROWE, 1968). ATP was found to be more effective in this series of experiments. The changes in coronary flow, heart rate and amplitude produced by 1·5 μM adenosine are shown in Fig. 5. 1·5 μM ATP had similarly small effects on heart rate (increased by 10 per

cent, mean of four observations) and amplitude of contraction (increased by 18 per cent). The effect of ATP on coronary flow, however, was considerable; the flow was increased from the mean control level of 8·1 ml per min to 16·2 ml per min.

Both adenosine and ATP produced an initial decrease in heart rate and amplitude of contraction. This effect is shown for adenosine in Fig. 5; 1·5 μM ATP produced smaller and more shortlived decreases in both parameters.

The onset of response to adenosine and ATP was slower than for the other vasodilators, taking 3–4 min to reach a maximum level. *Chironex* toxin in these experiments was therefore added 4 min after the introduction of the vasodilator. The effect of the toxin in the presence of adenosine is illustrated in Fig. 5. The percentage reduction in coronary flow and heart

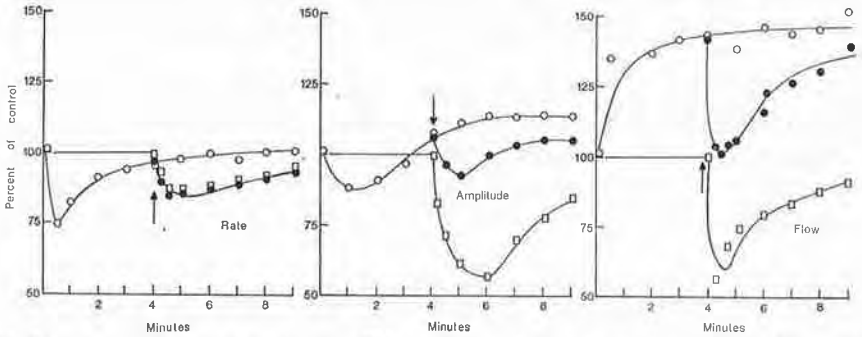

FIG. 5. EFFECT OF A STANDARD DOSE OF *Chironex* TOXIN IN THE PRESENCE OF 1·5 μM ADENOSINE. Adenosine is added at zero time; toxin is added at the arrow. Percentage changes after addition of toxin in the presence of adenosine are calculated using the heart rate, amplitude and coronary flow levels observed 4 min after addition of adenosine as 100 per cent values. □——□ 0·03 'mouse units' toxin, normal perfusion fluid; ○——○ 1·5 μM adenosine added to perfusion fluid at zero time; ●——● 0 03 'mouse units' toxin in presence of 1·5 μM adenosine.

rate was not significantly altered by the presence of either adenosine or ATP. The amplitude change, however, was reduced in both cases. In the presence of 1·5 μM ATP the reduction in amplitude after the toxin was 12 per cent; the reduction due to the toxin alone was 45 per cent. Thus these compounds can partially antagonize the negative inotropic effect of the toxin, but are relatively ineffective antagonists of the negative chronotropic effect and the coronary vasoconstriction.

In an attempt to characterize the rate and amplitude changes further, a work-time relationship was investigated. Previous workers have used the product of isometric tension and heart rate (BRINK *et al.*, 1969) or the product of peak systolic pressure and heart rate (MONROE and FRENCH, 1961) to give a measure of cardiac contractility. In the present instance the product of heart rate and work done by the heart (mass of load × peak amplitude of contraction) was used. The results of this procedure (Table 2) suggest that the inability

TABLE 2. EFFECT OF *Chironex* TOXIN AND VASODILATORS ON THE WORK-TIME FUNCTION (g per cm per min) OF THE PERFUSED HEART

	Control	Epinephrine 0·3 μM	Adenosine 1·5 μM	ATP 1·5 μM
Control	2247	4036	2510	2925
After toxin	1133	4520	1645	1865
Per cent change after toxin	−49·5	+12	−35	−36

of the heart to maintain its work level in the presence of the toxin is not greatly altered by adenosine or ATP but further illustrate the protective action of the catecholamines.

DISCUSSION

The isolated, perfused heart has been used in this series of experiments to examine further the cardiotoxic effects of *Chironex* toxin (FREEMAN and TURNER, 1969). Although the preparation is somewhat artificial in nature (OPIE, 1965) the results showed a reasonable correlation with those observed in the intact animal. Bradycardia followed a similar time course in both preparations and high levels of toxin resulted in irregular rhythms in both intact and isolated hearts. In addition, the reduction in amplitude of contraction in the isolated heart is consistent with the decrease in cardiac output which occurs in the whole animal. It is interesting to note that these effects occur in the isolated heart in the absence of neural control; this is in agreement with the concept of a direct action of the toxin on the heart (FREEMAN and TURNER, 1969; ENDEAN *et al.*, 1969).

The decrease in coronary flow rate produced by *Chironex* toxin is of considerable interest. If this is present in the intact animal it would combine with the poor venous return (FREEMAN and TURNER, 1969) and inadequate ventilation to cause hypoxic changes in the myocardium. In the isolated heart the decrease in coronary flow at the standard dose of toxin (0·03 'mouse units') is too brief to produce a significant degree of hypoxia; however, at doses comparable to those which are lethal in the whole animal, the decrease in flow is prolonged and could reasonably influence the rate and amplitude changes.

The vasodilator drugs were employed in an attempt to counteract the toxic response and in particular to reduce the coronary constriction. Coronary flow is increased by catecholamines either by a direct effect on the coronary vessels or by a secondary effect due to stimulation of cardiac metabolism (GLOMSTEIN *et al.*, 1967; ROWE, 1968); adenosine and ATP produce coronary vasodilatation by a direct action on either the vasomotor nerves or the vessel wall (ROWE *et al.*, 1962). The mechanism of action of serotonin has not been clarified (ROWE, 1968). All of these compounds increased coronary flow in the control situation but only the catecholamines antagonized the flow rate changes produced by the toxin.

The reduction in coronary flow produced by *Chironex* toxin is most likely to be a direct constrictor effect on the coronary vessels. The ineffectiveness of adenosine and ATP in antagonizing the coronary constriction may be due to a different site of action of these compounds and *Chironex* toxin; adenosine and ATP produce dilation at the capillary level (ROWE *et al.*, 1962; ROWE, 1968) while *Chironex* toxin and the catecholamines may both act at the arterial or arteriolar level. The toxin, however, does not appear to affect either alpha or beta adrenergic receptors as blockade of these produced no significant change in the toxin response.

The cause of the decrease in contraction amplitude and heart rate produced by the toxin cannot be well defined from these experiments. With large toxin doses hypoxia is likely to be a contributing factor to these changes, but the occurrence of the effects at low toxin doses where hypoxia is absent requires the presence of another factor. It was suggested previously (FREEMAN and TURNER, 1969) that the toxin exerted a non-specific lytic effect on cell membranes; this could affect both the conducting system of the heart and ventricular contractility and thus provide an explanation of the results. The improvement in amplitude of contraction produced by adenosine, ATP and the catecholamines in the presence of the toxin suggests that these compounds may improve the metabolic status of the intoxicated heart.

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Maculotoxin, a Potent Toxin Secreted by *Octopus maculosus* Hoyle

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Reprinted from TOXICOLOGY AND APPLIED PHARMACOLOGY, Vol. 16, No. 3, May 1970
 Printed in England

TOXICOLOGY AND APPLIED PHARMACOLOGY 16, 681–690 (1970)

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Received July 2, 1969

Maculotoxin, a Potent Toxin Secreted by *Octopus maculosus* Hoyle. FREEMAN, SHIRLEY, E., and TURNER, R. J. (1970). *Toxicol. Appl. Pharmacol.* **16**, 681–690. The posterior salivary (venom) glands of the blue-ringed octopus, *Octopus* (*Hapalochlaena*) *maculosus* Hoyle have been shown to contain a neurotoxin which is pharmacologically very similar to tetrodotoxin and saxitoxin. The toxin, which has been called "maculotoxin," causes hypotension, bradycardia, and respiratory paralysis in rabbits and rats. The hypotension can be temporarily reversed with *l*-epinephrine or *l*-norepinephrine. Evidence is presented which suggests that respiratory failure after intravenous injection is due to blockade of muscular nerve axons. The toxin blocks transmission in the sciatic nerve of the toad and the rat, and at low dose levels appears to have neuromuscular blocking activity. At higher dose levels the muscle membrane also becomes inexcitable. It is without significant effect on the acetylcholine-induced contraction of the chronically denervated rat diaphragm, the toad rectus abdominis, or the guinea pig ileum.

Animals can be resuscitated after a marginal lethal dose by artificial ventilation alone, provided this is instituted before hypoxia becomes severe.

The small, blue-ringed octopus *Octopus* (*Hapalochlaena*) *maculosus* Hoyle, has been responsible for a number of cases of temporary paralysis among people who have handled the animal on Australian beaches. Victims have described similar symptoms, which include weakness, nausea, and respiratory difficulty. There have been 3 fatalities. Death has been ascribed to respiratory failure due to flaccid paralysis, with a time of onset of 5–10 min. Sutherland and Lane (1969) have recently discussed medical aspects of the envenomation.

This species of octopus appears to be moderately abundant in Australian coastal waters during the summer months. It is not, however, a serious hazard to swimmers since it is not aggressive, and has moreover a very small "beak," which makes a penetrating injection of venom into a victim difficult. As victims are usually unaware of the envenomation until symptoms develop, it is possible that bites occurring under water may go unnoticed, as the venom would wash out of a shallow wound. The venom is secreted by the large posterior salivary glands of the octopus and appears to be used to immobilize the small crabs that are its prey.

The present report of the pharmacology of the venom is an extension of a preliminary account (Simon *et al.*, 1964), in which the venom was found to be neurotoxic

and to block neuromuscular transmission. A study of the chemistry of the toxin will be published elsewhere.

It became evident in the course of the present investigation that the toxin, which has been tentatively termed "maculotoxin," is pharmacologically very similar to tetrodotoxin and saxitoxin. The occurrence of these toxins in such a phylogenetically diverse range of organisms is of some interest. Their presence in dinoflagellates, cephalopods, and vertebrates suggests that they may be more widely distributed than is now known.

METHODS

Collection and preparation of toxin. The authors are indebted to members of the public for the collection of octopods from coastal waters. Early in the investigation, the animals were maintained in an aquarium until required, and fed daily on small crabs. However, on several occasions they died when the seawater was changed. apparently due to the presence of a pollutant. Thereafter the posterior salivary glands were dissected out soon after capture, and kept in liquid nitrogen or in the deep freeze at −15°.

Glands from different animals contained widely differing amounts of toxin per milligram of gland tissue; in particular it was noted that animals in poor condition frequently contained virtually no toxin. Consequently the present study was carried out on an extract made from the pooled glands of 11 animals. Tissue, 3.2 g, was homogenized in 30 ml of distilled water, and the debris was centrifuged. The water-soluble toxin was partially purified by filtration through Sephadex G-25 columns buffered to pH 8.0 with an isotonic tris-buffered saline solution. The lethal fraction was eluted from the column in a discrete peak, which contained approximately 70% of the lethality of the original extract, and consequently must represent the major or only lethal component.

It was possible by this procedure to separate the lethal fraction, which does not absorb in the ultraviolet region, from a contaminant which absorbs at 274 nm, (Simon *et al.*, 1964). The earlier study was also complicated by the fact that acetone extracts of the glands were used as starting material. It has now been shown that the toxin is only partially soluble in acetone.

Figure 1 illustrates the separation of maculotoxin from the ultraviolet-absorbing material by Sephadex G-25 filtration. The elution volume of saxitoxin is also shown for comparison. It is clear that maculotoxin has a similar size and molecular configuration to saxitoxin.

Pharmacologic testing of toxin. The lethality of the toxin for mice (23–25 g) was determined by injection of 0.1 ml of material with a Hamilton 100-μl syringe into the tail veins of groups of 5–6 mice, or by ip injection of the toxin diluted to 1 ml with water. It was found that the iv mouse unit was comparable to the ip mouse unit, as defined by workers with saxitoxin and tetrodotoxin (Schantz, 1960; Kao, 1966).

The effect of the toxin on various physiological parameters was tested using New Zealand rabbits (2.2–3.2 kg) and Wistar rats (300–320 g). The rabbits were anesthetized with urethane and maintained with chloralose injected into the marginal ear vein. Rats received 50 mg/kg of pentobarbital ip. When experiments were prolonged, anesthesia was maintained with supplementary doses.

Arterial and venous pressures were monitored with Statham pressure transducers; the venous catheter was placed in the jugular vein and passed down to the level of the atrium. Lead II electrocardiograms were recorded; heart rate was recorded with a cardiotachometer. Abdominal respiratory movement was recorded with a colloidal carbon-in-rubber pneumograph; body temperature was monitored with a rectal thermistor. All parameters were monitored on a Beckman type R dynograph recorder.

In other experiments, the phrenic nerve of the rabbit was exposed in the neck and platinum electrodes were placed on it. A myograph needle was placed in the diaphragm muscle, and the respiratory activity of both muscle and nerve was displayed on a dual beam oscilloscope and photographed on moving film.

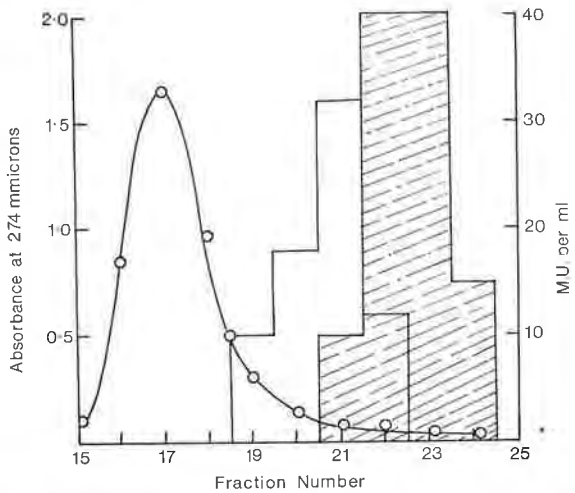

FIG. 1. The Sephadex G-25 separation of maculotoxin from an aqueous extract of *Octopus maculosus* gland. Maculotoxin (hatched area) is well separated from an ultraviolet absorbing contaminant (○—○). Saxitoxin (unhatched area) was run through the column on the succeeding day, and relative to the Dextran Blue marker had a slightly smaller elution volume. Fractions (5 ml) are numbered from 1 after the void volume. Bed volume was approximately 200 ml.

Isolated organ preparations. The effect of the toxin was tested on the isolated phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946), the chronically denervated rat diaphragm (Freeman and Turner, 1969), the toad rectus abdominis preparation, the isolated guinea pig ileum, the isolated toad (*Bufo marinus*) sciatic nerve (Kao and Fuhrman, 1963), and the isolated perfused guinea pig heart (Turner and Freeman, 1969).

RESULTS

Effects in Mice

After intravenous injection of the toxin, mice became agitated, ataxic, and the hind limbs splayed and became paralyzed. Breathing was labored and ceased after 60–90 sec. Terminally there were brief but violent convulsions. At autopsy the heart was found to be beating irregularly. There was no macroscopically evident pathology. The dose-mortality curve was extremely steep. The relationship between the iv minimum

lethal dose and the ip "mouse unit" as calculated by workers with other neurotoxins may be seen from Fig. 2, in which the logarithm of dose is plotted against the reciprocal of the time to death after ip injection. One iv mouse unit caused death in approximately 20 min when given by the ip route. Therefore, if the mouse unit (M.U.) is defined as the dose required to kill a mouse in 10 min after ip injection (Kao, 1966), then approximately 1.8 iv M.U. are equivalent to 1 ip unit. Data in the present study are expressed in terms of the iv unit.

Figure 2 also shows the relationship between the iv and the ip unit for tetrodotoxin. It may be seen that the data for the two toxins coincide, suggesting a close similarity between them. Further, death after tetrodotoxin followed an identical pattern to that

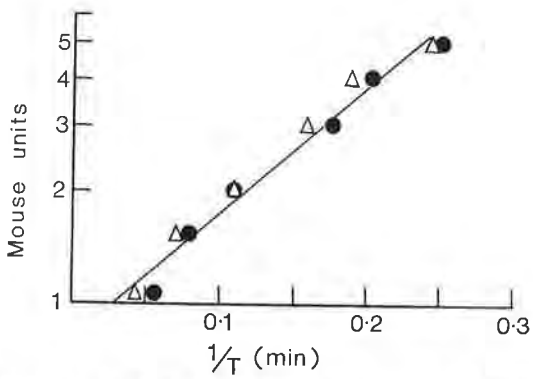

FIG. 2. Dose-death time relationships. The logarithm of dose (iv M.U.) is plotted against the reciprocal of time to death in minutes after intraperitoneal injection. Maculotoxin (△—△) and tetrodotoxin (●—●). Six to 8 mice were used at each dose level.

seen after maculotoxin. Konusu *et al.* (1968) have shown that the time to death data after saxitoxin yield a significantly different slope from tetrodotoxin data. Therefore, maculotoxin appears to resemble tetrodotoxin more closely than saxitoxin.

Effects in Rabbits

Arterial and venous pressure, respiratory rate and depth, and electrocardiograms (ECG) were recorded as described in the Methods section. Anesthetized rabbits proved to be very sensitive to the toxin: iv doses of 3–8 M.U./kg were sufficient to cause hypotension, bradycardia, and respiratory depression. Experiments were designed so that a nonlethal dose preceded a lethal dose by some 30–40 min.

The systolic arterial pressure was reduced from the control level of 110–135 mm Hg (5 animals) to 50–65 mm Hg over a 3-min period. There was no change in venous pressure or pulse pressure. The heart rate fell from 300–330 beats per min to 250–270 per minute over the same period; the ECG showed no other abnormality. These effects persisted for 20–30 min. The respiratory depression was rather more variable; the rate fell from an average of 60 per minute to 35–48 per minute, and there was a slight decrease in amplitude. This depression was more transient than the cardiovascular effects.

The toxin appeared to have a cumulative effect, since a second dose of similar size given after all symptoms had subsided was usually sufficient to cause respiratory failure

(see also Simon *et al.* 1964). The arterial pressure fell concurrently with the respiratory difficulties, and ranged from 30 to 45 mm Hg when breathing ceased. Venous pressure rose, and the ECG showed marked bradycardia and T-wave enlargement.

Effects in Rats

Rats showed essentially similar symptoms after injection of the toxin into a tail vein. Approximately 15 M.U./kg toxin lowered the systolic arterial pressure from the control level of 135–180 mm Hg (5 animals) to 75–95 mm Hg. The venous pressure was unchanged or rose slightly concurrently with the arterial pressure fall. The heart rate fell from 390–400 beats per minute to 280–320. The respiratory rate fell from the usual level of 66–80 per minute to 55–60 per minute over a 3-min period. There was occasionally a transient increase in amplitude immediately after injection of the toxin.

Hypotension and bradycardia persisted for more than 30 min in the rat, although after this interval respirations were again normal. A second dose of 24–40 M.U./kg toxin brought about respiratory arrest in 1–4 min. Respirations slowed immediately following injection of the toxin, and the amplitude declined. In instances where death was prolonged over several minutes, respirations became very slow, prolonged, and gasping. Occasionally a period of apnea preceded a final respiratory effort. The arterial pressure fell concurrently with these changes, and averaged 50 mm Hg when respirations ceased. Venous pressure rose as arterial pressure fell, and the ECG showed hypoxic abnormalities.

The observation that hypotension after maculotoxin injection in both the rabbit and rat always accompanied or preceded respiratory depression suggested a closer similarity with tetrodotoxin than with saxitoxin (Kao and Nishiyama, 1965). We confirmed that respiratory failure due to tetrodotoxin in the rat was accompanied by a more profound hypotension than was seen after saxitoxin.

Effects of Catecholamines on the Hypotensive Response

In both rabbits and rats hypotension due to a nonlethal dose of toxin was temporarily reversed by epinephrine or norepinephrine. Experiments were designed so that a test dose of the catecholamine was given before and after a nonlethal dose of toxin.

In the rat 1.5 μg/kg of *l*-norepinephrine bitartrate raised the systolic arterial pressure to approximately 200 mm Hg without changing heart rate or venous pressure. A similar dose some 5 min after 15 M.U./kg maculotoxin also raised the systolic arterial pressure to 200 mm Hg, although the arterial pressure had been reduced by maculotoxin. Heart rate and venous pressure were again unchanged.

The arterial pressure was raised by a similar amount by *l*-epinephrine bitartrate (2 μg/kg) without change in heart rate but with a slight increase in venous pressure. After treatment with maculotoxin, epinephrine raised the depressed arterial pressure to the same level, but in this instance there was a significant increase in heart rate. Comparable experiments were carried out with tetrodotoxin, with closely similar results. Rabbits responded to doses of 2.5 μg/kg norepinephrine or 7 μg/kg epinephrine in the same way. These results may be compared with those of Kao and Fuhrman (1963).

Since the terminal drop in arterial pressure is precipitous and is likely to contribute to the fatal outcome, norepinephrine (2.5 μg/kg) was given to rabbits during the period

of respiratory depression prior to terminal apnea. It was ineffective however in preventing the terminal fall in arterial pressure, and moreover precipitated a cardiac arrhythmia.

Effect of Maculotoxin on Phrenic Nerve Activity

The respiratory depression induced by maculotoxin in the rabbit was further examined by monitoring the respiratory volleys passing down the phrenic nerve, in

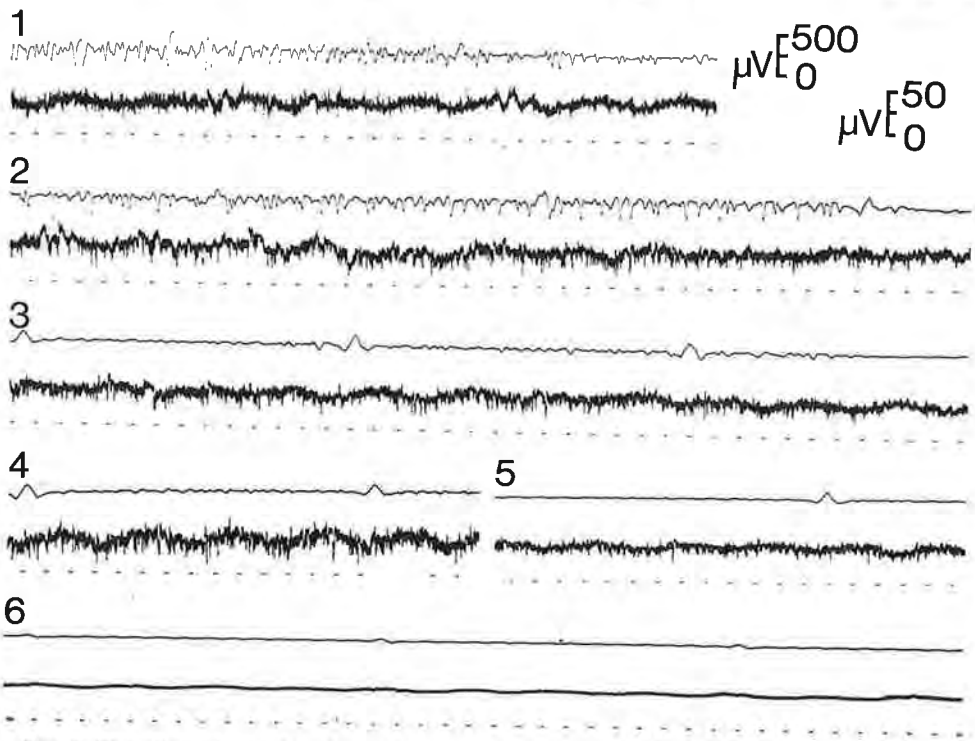

FIG. 3. The effect of maculotoxin on the electrical activity of the phrenic nerve and diaphragm. Panel 1, part of a control respiration; panel 2, a prolonged respiration after 5 M.U./kg maculotoxin; panel 3, diminished diaphragm activity after a further 7 M.U./kg; panel 4, increased phrenic activity which had little effect on the diaphragm; the ECG showed clearly; panel 5, the onset of failure of phrenic volleys; panel 6, electrical silence at death. The ECG caused a barely detectable deflection in this trace. Time marker = 0.02 sec.

conjunction with the output of a myograph needle placed in the diaphragm. The results of one such experiment are illustrated in Fig. 3.

The anesthetized animal had a respiratory rate of 50 per minute and an arterial pressure of 120 mm Hg. Inspiration lasted 0.5 sec and the respiratory pause was 0.7 sec. Maculotoxin (5 M.U./kg) reduced the arterial pressure to 60 mm Hg and the respiratory rate to 35 per minute. Diaphragmatic activity lasted approximately 1.2 sec, and the respiratory pause was 0.5 sec in duration. The animal appeared to be adequately ventilated, and during the next 15 min the arterial pressure rose to 105 mm Hg, and the respiratory rate returned to the control level. A second slow iv injection of

5 M.U./kg was given at this time. Arterial pressure fell to 60 mm Hg, and respirations were reduced to approximately 30 per minute. The diaphragm showed almost continuous electrical activity, although both amplitude and frequency of muscle potentials was somewhat reduced (see Fig. 3, panel 2). There was a concurrent increase in amplitude of phrenic volleys. However, the animal survived, and a final dose of 7 M.U./kg was necessary to abolish respirations; apnea occurred 3 min after this injection. Initially breathing became largely intercostal, coincident with a decline in the amplitude of the myograph output. The phrenic volleys increased in amplitude, although they produced only a ripple on the myograph trace. Finally they diminished in frequency and amplitude until at death there was electrial silence. These effects are illustrated in Fig. 3, panels 3–6. After respirations ceased a tetanic stimulus applied to the phrenic nerve produced a weak diaphragmatic contraction.

Neuromuscular Blocking Activity of Maculotoxin

Maculotoxin brought about complete blockade of the isolated phrenic nerve-diaphragm preparation of the rat at concentrations equal to or greater than 0.15

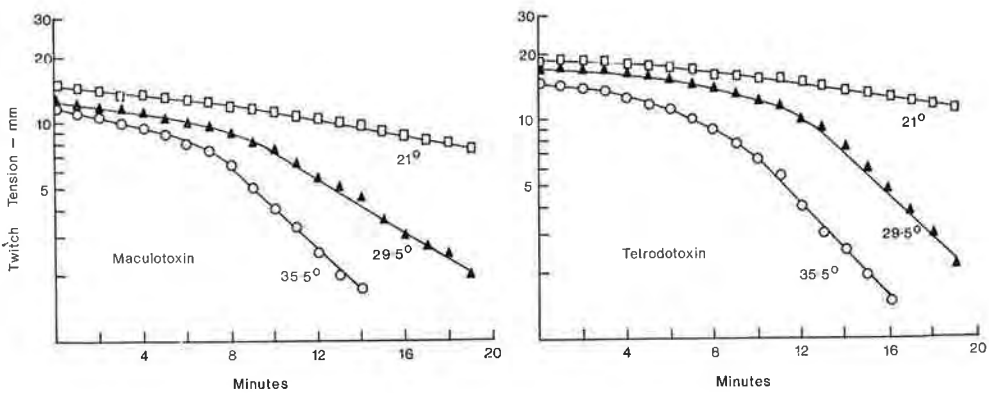

FIG. 4. The onset of neuromuscular blockade after equivalent doses of maculotoxin and tetrodotoxin at 21–35.5°. Semilogarithmic coordinates; 20 mm twitch tension = 18.2 g.

M.U./ml. The muscle remained responsive to direct stimulation after transmission was blocked, but the threshold was raised. A 5- to 10-fold increase in stimulating voltage was required for maximum tension development. High doses or prolonged treatment rendered the muscle inexcitable. Shortly before transmission failed, the preparation was able to sustain a 10-sec tetanus. The rate of onset of blockade was independent of the frequency of nerve stimulation over the range 0.1–1.0 per second.

Neuromuscular blockade was characterized by a long latent period when twitch tension declined very little, followed by a rapid decline in tension which followed an exponential time course. Figure 4 illustrates the onset of blockade. It may be recalled that Kao and Nishiyama (1965) found a similar latency when measuring the decline in end plate potential after saxitoxin treatment. Recovery was prolonged; it was found necessary to wash preparations for upward of 90 min to avoid residual effects of retained toxin. Because of this effect, minimum doses consistent with complete blockade were always used.

The rate of development of blockade showed the same steep dose dependency as was found for lethality in the intact animal.

Both maculotoxin and tetrodotoxin were more effective as neuromuscular blocking agents at high temperature than at low temperature. Figure 4 illustrates the effect of temperature on equivalent doses of maculotoxin and tetrodotoxin. Toxin doses that were almost without effect at 21° caused complete blockade in 16–17 min at 35.5°. In a further series of experiments it was found that equivalent doses of both toxins (calibrated as mouse units) caused neuromuscular blockade at a similar rate, when both the length of the latent period and the time to half decay of tension were compared.

Effects on the Nerve Action Potential

As was found previously (Simon *et al.*, 1964) maculotoxin caused a reversible blockade of the desheathed toad sciatic nerve. Dose levels of 0.2 M.U./ml caused widening of the compound action potential and a decrease in conduction velocity prior to transmission block. Rat sciatic nerve was similarly blocked by maculotoxin. The rat sciatic nerve was difficult to desheath, so the relatively fine peroneal branch was used in these experiments.

Effects on the Chronically Denervated Rat Diaphragm and Toad Rectus Abdominis Preparation

Acetylcholine dose response curves were plotted for the chronically denervated rat diaphragm (Freeman and Turner, 1969) and were repeated after the addition of maculotoxin to the organ bath. Levels of maculotoxin ranging from 0.3 to 1.0 M.U. per millilitre produced no direct effect on the diaphragm, neither was the acetylcholine dose-response curve altered by the presence of the toxin. The rectus abdominis preparation was similarly unaffected by maculotoxin. These two preparations are also insensitive to tetrodotoxin and saxitoxin (Freeman and Turner, 1969; Kao, 1966). Like these toxins, maculotoxin (15 M.U./ml) was also found to be without anticholinesterase activity (H. D. Crone, personal communication).

Effects on Guinea Pig Ileum Preparation

The maculotoxin preparation caused a slight contraction of the ileum preparation. This effect was blocked by diphenhydramine (10^{-7} g/ml) but was unaffected by atropine or lysergic acid diethylamide. It is likely that the gland extract contained some histamine, which was not separated from it by the process of Sephadex filtration. However, the presence of 0.25 M.U. of maculotoxin per milliliter did not affect the acetylcholine-induced contraction of the ileum over a wide concentration range.

Isolated Perfused Guinea Pig Heart

Maculotoxin (10 M.U.) was injected into the perfusion system just proximal to the heart. This resulted in a marked reduction in coronary flow rate coincident with an increase in heart rate over the first 30 sec followed by a return of both to control levels after 1 min. Isotonic contraction amplitude increased over the first min and then decreased to about 30% of the control value for 2–3 min with a slow return to normal over the following 5 min. Doses less than 5 M.U. were without any observable effect on the heart.

The cardiac effects of maculotoxin are probably insignificant in the overall toxin response since the amounts required to affect the heart are more than sufficient to cause rapid death through hypotension and respiratory paralysis.

Resuscitation of Animals

Since the cause of death after maculotoxin envenomation appears to be respiratory paralysis which is associated with profound hypotension, attempts to resuscitate rats and rabbits were made by respiring them with a ventilation pump.

It was found possible to revive animals only after marginally lethal doses of maculotoxin or tetrodotoxin, and then only if the pump was started before respiratory depression became severe. Animals that were breathing but cyanotic, and that had a systolic blood pressure of less than 40 mm Hg, had a poor prognosis. In some cases artificial ventilation was effective in raising the arterial pressure to approximately 60 mm Hg and in restoring a near normal ECG pattern, but spontaneous respiration did not occur even after ventilating the animal for 120 min.

When successful, ventilation had to be continued for upward of an hour after the return of spontaneous respiratory efforts in order to maintain the animal. This slow recovery of the paralyzed musculature is consistent with the slow washout of toxicity from the isolated diaphragm preparation.

DISCUSSION

The pharmacology of the toxin of *O. maculosus* as described in the present communication is consistent with the earlier report (Simon *et al.*, 1964). The present investigation suggests that the toxic effects are due to only one compound, whereas previously there appeared to be two. The work of Sutherland and Lane (1969) also suggests that there are two chromatographically distinct toxins. The elucidation of this point must await the results of studies now in hand, and will be reported at a later date.

Maculotoxin, whether it be one or two molecules, resembles tetrodotoxin and saxitoxin in that death after envenomation is due to respiratory paralysis accompanied by profound hypotension. Maculotoxin preparations that have been calibrated as mouse units show quantitatively similar effects to tetrodotoxin when compared in a variety of pharmacological tests. The occurrence of hypotension at doses just sufficient to produce respiratory depression strengthens the evidence that the toxin is more closely related to tetrodotoxin than to saxitoxin (Kao and Nishiyama, 1965).

The failure of respiration in death after maculotoxin injection was characterized by a loss of diaphragmatic activity which preceded the failure of phrenic nerve volleys. Indeed for a short time these increased in amplitude, no doubt due to the effect of inadequate ventilation on the respiratory centre. However the increase in phrenic activity was at no time comparable to that seen after the administration of a neuromuscular blocking agent such as gallamine or *d*-tubocurarine. The rate of onset of effects probably reflects the rate at which the toxin is carried to the various organs by the circulation. The vascularity of the diaphragm may permit of muscular axonal block before this is evident in the phrenic nerve. Any central effects are also likely to be delayed by diffusion across the blood-brain barrier. Cheng *et al.* (1968) have discussed such effects in regard to respiratory failure after tetrodotoxin.

The lack of effect of maculotoxin on either the nicotinic or muscarinic acetylcholine receptors, as exemplified by the denervated diaphragm, the toad rectus abdominis and the guinea pig ileum, respectively, also confirms its similarity to tetrodotoxin and saxitoxin. Local anesthetic drugs such as procaine, which block axonal transmission and cause hypotension, also have anticholinergic effects.

Resuscitation by ventilating an intoxicated animal with a respiration pump required that the animal have a marginal lethal dose, that ventilation be instituted before cyanosis became evident, and that it be prolonged for some time after the return of spontaneous respiratory movements. Although the anesthetized animal cannot be compared with man, this finding is in line with the clinical observation that respiratory paralysis may last for upward of 8 hours. In his discussion of tetrodotoxin poisoning, Li Shih-Chen (1596) noted in The Great Herbal that "the poisoning no remedy can relieve." It would appear to be true today that there is no specific antidote for the neurotoxins related to tetrodotoxin.

ACKNOWLEDGMENTS

The authors are indebted to Dr. E. J. Schantz, Fort Detrick, Maryland, for a gift of saxitoxin. We should also like to thank the Nuffield Foundation for assistance.

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THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 174, No. 3

Printed in U.S.A.

FACILITATORY DRUG ACTION ON THE ISOLATED PHRENIC NERVE-DIAPHRAGM PREPARATION OF THE RAT

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Accepted for publication May 10, 1970

ABSTRACT

FREEMAN, SHIRLEY E. AND R. J. TURNER: Facilitatory drug action on the isolated phrenic nerve diaphragm preparation of the rat. J. Pharmacol. Exp. Ther. **174**: 550–559, 1970. The action of facilitatory drugs was studied in the phrenic nerve-diaphragm preparation and the chronically denervated diaphragm of the rat. The latter was used as a model of the postsynaptic receptor. The drugs were tetrahydro-4-aminoacridine and a series of hydroxyanilinium compounds which included edrophonium. The drugs caused twitch potentiation and spontaneous activity in the intact preparation; these effects were depressed by temperature reduction, low Ca^{++} solutions or high Mg^{++} solutions. The acetylcholine contraction of the denervated diaphragm was potentiated by all drugs except 3-hydroxyphenyltriethylammonium. The acetylcholine depolarization was similarly affected. This potentiation was suppressed by raised levels of Ca^{++} or Mg^{++}. Interactions between the facilitatory drugs and succinylcholine are described. Succinylcholine abolished twitch potentiation of the intact preparation at low concentrations; only 3-hydroxyphenyldiethylmethylammonium proved to be an effective antagonist of succinylcholine blockade. Facilitation in the intact junction appears to be largely a presynaptic effect.

The phenomenon of drug-induced facilitation of the indirectly elicited muscle twitch has been studied by a number of authors. Both pre- and postsynaptic sites have been implicated in the facilitatory process; the greater emphasis has been placed on presynaptic actions (Werner, 1960; Standaert and Riker, 1967; Karczmar, 1967).

The occurrence of this phenomenon in the isolated phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946) permits a study of the effects of variation in temperature and in the external ionic environment on the facilitatory process. In this way some indication as to the relative importance of pre- and postsynaptic factors has been obtained. At the same time the acetylcholine (ACh) receptor of the chronically denervated diaphragm has been used as a model of the postsynaptic ACh receptor (Freeman and Turner, 1968a, 1969). Although the receptor induced in the diaphragm by denervation is known to differ quantitatively from the innervated postsynaptic receptor with regard to interactions with bisquaternary compounds (Beránek and Vyscočil, 1967; Vyscočil and Beránek, 1968), this preparation has been shown to offer a reasonable method for examining ACh-receptor interaction and has certain advantages over the intact junction.

First, this receptor is easy to approach experimentally; drug concentrations and ionic levels can be maintained as required. Second, it contains acetylcholinesterase only in the region of the degenerating synapse (Eränkö and Teräväinen, 1967).

The effects of four facilitatory drugs have been determined on the innervated and the denervated preparation. In addition, interactions with succinylcholine (SCh) have been studied to determine the role of facilitatory processes in the relief of SCh blockade.

The drugs studied are a series of hydroxyanilinium compounds which includes edrophonium and the structurally unrelated compound tetrahydro-4-aminoacridine (tacrine). The study offers evidence of pre- and postsynaptic actions of these compounds and permits tentative conclusions as to the structural features of the series.

Received for publication February 4, 1970.

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METHODS. Male, hooded rats of the Wistar strain weighing from 225 to 270 g were used in this study. The animals were stunned and exsanguinated; both left and right hemidiaphragms were dissected out and set up in separate organ baths. Isometric tension developed by the muscles in response to direct or indirect stimulation was measured with Statham UC2 transducing cells and a Beckman type R Dynograph recorder. Preparations were stimulated with 0.1-msec rectangular pulses of supramaximal voltage at 9/min.

Membrane potentials were measured by a technique similar to that of Lüllmann and Reis (1967). Micropipettes were made from 2.5-mm glass tubing (Micropet, Clay Adams, Parsippany, N.J.) and were filled with 3 M KCl under reduced pressure. Tip resistances were 6 to 20 megohms, and tip potentials were less than 10 mV (Thesleff, 1963). The micropipette was attached by a Perspex holder to a Singer micromanipulator and was connected through a Ag/AgCl electrode to a preamplifier. A calibrated voltage source between the organ bath and earth permitted rapid measurement of the membrane potental by acting as an opposing potentiometer. The potential difference was read when the current was zero. Preamplifier output was fed into an oscilloscope and a Beckman type RP Dynograph recorder. Membrane potentials were measured with an accuracy of ± 0.5 mV.

The diaphragm muscle was pinned to a silicone rubber block (Rhodorsil) which covered the base of the organ bath.

Denervation was carried out under pentobarbital anesthesia by evulsion of the left phrenic nerve in the neck (Freeman and Turner, 1968a, 1969). The animals were maintained postoperatively for from 8 to 15 days to allow the nerve to degenerate.

Depolarization of denervated diaphragm muscles by ACh was measured after the ACh-induced contraction had passed, since the contraction displaced the micropipette from the muscle cell. We confirmed the observation of Lüllmann and Reis (1967) that depolarization persisted after relaxation.

The nutrient solution used has been described elsewhere (Freeman, 1968a). The bath temperature was monitored continuously with a thermistor. Unless otherwise stated, experiments were carried out at 29 ± 0.2°C.

The facilitatory drugs used were tetrahydro-4-aminoacridine hydrochloride (tacrine), 3-hydroxyphenyldimethylethylammonium chloride (edrophonium), 3-hydroxyphenyldiethylmethylammonium bromide (3-OHPEt$_2$MeA) and 3-hydroxyphenyltriethylammonium bromide (3-OHPTEA). Succinylcholine chloride and acetylcholine chloride were dried before weighing out for stock solutions. These were kept at −20°C until required.

RESULTS. *Facilitatory drug action on neuromuscular transmission.* Facilitation of the isometric twitch occurred with all the compounds studied; it developed over a period of 5 to 8 minutes after addition of the drug to the organ bath and persisted for the time of exposure of the preparation to the drug. In the case of the three hydroxyanilinium compounds the effect subsided when the preparation was washed; however, potentiation due to tacrine persisted for upwards of 40 minutes after washout at doses less than 5×10^{-6} M, and this effect was virtually irreversible at higher doses. All compounds with the exception of 3-OHPTEA caused some degree of spontaneous twitching. Such twitching was particularly marked with tacrine (see fig. 1, panels 1 and 2), although it varied among preparations.

As has been reported in experiments *in vivo* (Kuperman *et al.*, 1961; Blaber and Karczmar, 1967b) the drug concentration for optimal facilitation varied over a wide range; in our experiments this extended from 5×10^{-6} M for tacrine to 10^{-3} M for 3-OHPTEA. Figure 2 shows dose-response curves for tacrine, edrophonium and 3-OHPEt$_2$MeA. The slopes of the linear portions of the dose-response curves were not significantly different at the 5% probability level. The curve for 3-OHPTEA resembled that of the other two hydroxyanilinium compounds save that a 100-fold greater drug concentration was required. However, tacrine showed twitch depression at dose levels closer to those required for maximal twitch potentiation than did the hydroxyanilinium compounds.

Twitch potentiation after addition of each of the drugs was frequency dependent. It was maximal at stimulation rates between 6 and 12/min, and usually leveled off to the same contractile force as the untreated preparation at rates greater than 1/sec. The frequency dependence was tested in all instances with drug levels which produced optimal potentiation.

Temperature sensitivity. The facilitatory process was found to be sensitive to the temperature of the bathing solution. Lowering the temperature to 20°C abolished potentiation and spontaneous firing, which were maximal at 36°C. Experiments were carried out to estimate the apparent energy of activation (E_a) of the facilitatory process. Since the normal process of neuromuscular transmission is itself temperature sensitive, experiments were carried out so that

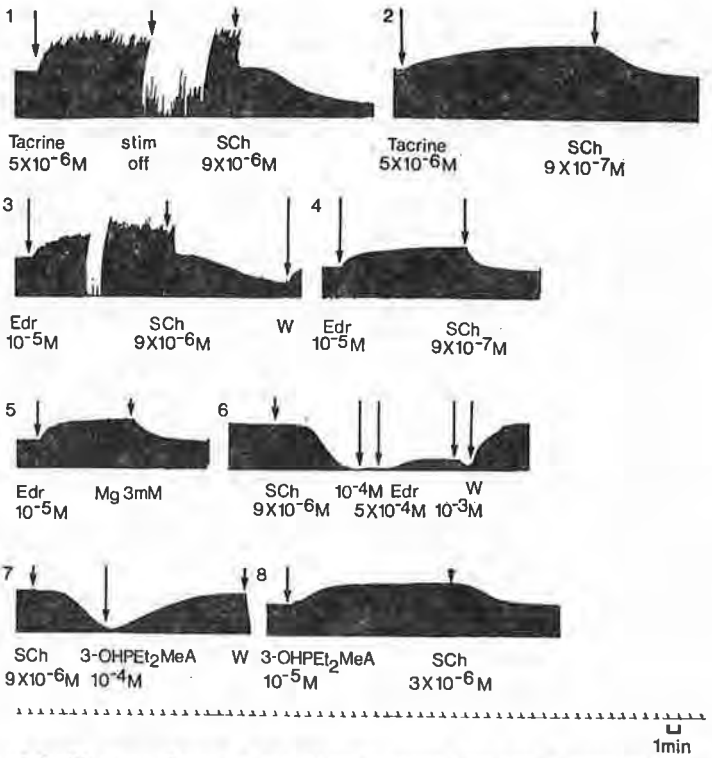

FIG. 1. Effects of facilitatory drugs on rat phrenic nerve-diaphragm preparations; interactions between these drugs and different levels of SCh. Panel 1, tacrine (5×10^{-6} M) was added at first arrows Stimulator was switched off for four minutes at second arrow. Twitch potentiation and spontaneou. activity were suppressed by SCh (9×10^{-6} M) added at third arrow. Panel 2, tacrine produced twitch potentiation but less spontaneous activity. SCh (9×10^{-7} M) suppressed potentiation without causing blockade. Panel 3, edrophonium (10^{-5} M) was added at arrow. Spontaneous activity and twitch potentiation were suppressed by SCh (9×10^{-6} M). Panel 4, twitch potentiation due to edophonium was blocked by SCh (9×10^{-7} M) without subsequent blockade. Panel 5, potentiation due to edrophonium was abolished by 3 mM Mg^{++}. Panel 6, SCh blockade was followed by the sequential addition of edrophonium (10^{-4} M, 5×10^{-4} M and 10^{-3} M); only 5×10^{-4} M significantly relieved blockade. Panel 7, SCh blockade was relieved by 10^{-4} M 3-OHPEt₂MeA. Panel 8, potentiation due to 3-OHPEt₂MeA (10^{-5} M) was suppressed by SCh (3×10^{-6} M). Twitch tension in the absence of drugs was 10 to 13 g; time marker = 1 minute.

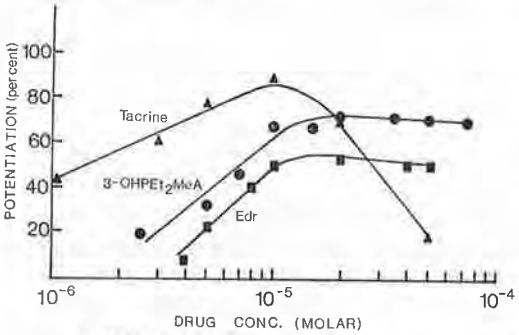

FIG. 2. The relationship between percent increase in twitch tension and drug concentration for tacrine (▲——▲), 3-OHPEt₂MeA (●——●) and edrophonium (■——■). Each point is the mean of from six to nine observations. Stimulation rate = 9/min. Semilogarithmic scale.

twitch tension was determined at each temperature before the addition of drug to the bath. In the case of tacrine, where the drug could only be washed out of the preparation very slowly, the twitch tension was determined over the temperature range from 20–36°C prior to drug addition; the temperature was then varied over the same range in the presence of the drug, and the percentage of twitch potentiation was determined. The concentration of tacrine was 5×10^{-6} M, and that of edrophonium was 10^{-5} M.

Results were plotted as the logarithm of the percent increase in tension against the reciprocal of the absolute temperature, and the apparent energy of activation, E_a, was derived from the Arrhenius equation with the statistically deter-

mined regression. Several assumptions are implicit in making such calculations: first, that potentiation is proportional to reaction rate; second, that one reaction in the sequence leading to potentiation is dominant and provides a rate-determining step; and third, that this reaction retains its dominance over the temperature range studied. Some support for the latter two assumptions is given by the fact that an analysis of variance showed that there was a highly significant linear correlation between $\log_{10}$ potentiation and the reciprocal of the absolute temperature.

For tacrine, $E_a = 25.2 \pm 1.2$ kcal mol^{-1} (95% confidence limits). For edrophonium, $E_a = 26.7 \pm 3.3$ kcal mol^{-1} (95% confidence limits). The figures are not significantly different at the 5% probability level. It is of interest to note the high values of E_a obtained and to compare them with the value of approximately 48 kcal mol^{-1} obtained by Hofmann *et al.* (1966) for E_a of the miniature end-plate potential frequency in the rat diaphragm.

Changes in external divalent cation levels. Twitch potentiation was sensitive to small changes in the divalent cation level of the bathing solution. Raising the Ca^{++} level to 3.0 or 4.5 mM was either without effect (tacrine) or increased the drug-induced potentiation (all hydroxyanilinium compounds). It was noted that 3.0 mM Ca^{++} increased twitch potentiation by the hydroxyanilinium compounds more at low drug levels than at optimal levels. That is, the dose-response curve in high Ca^{++} diverged from the control curve, and the drug level necessary for optimal potentiation was reduced.

On the other hand reduction in the Ca^{++} level to 0.38 mM (× 0.25 normal) completely abolished twitch potentiation in all instances. If the Ca^{++} level was reduced to 0.15 mM (× 0.1 normal), a slow blockade of neuromuscular transmission occurred (Freeman, 1968b). Addition of drugs to the organ bath during the course of the low Ca^{++} blockade caused varying effects. Tacrine (5×10^{-6} M) caused a transient relief of blockade lasting for one to two minutes; twitch tension then continued to decrease at the original rate. This effect was also found with edrophonium and 3-OHPEt$_2$MeA at concentrations up to 10^{-4} M. Higher drug levels increased the rate of onset of low Ca^{++} blockade. However 3-OHPTEA (10^{-4} M) either brought about a slight relief of low Ca^{++} blockade or halted its progress. In this regard this compound somewhat resembles the structurally related drug triethylcholine (Freeman, 1968b).

Reduction of the external Mg^{++} level to 0.1 mM (× 0.1 normal) was without effect on twitch potentiation in all instances. However, small increases in external Mg^{++}, which were in themselves without effect on twitch tension, completely abolished twitch potentiation. Raising external Mg^{++} from the usual level of 1 mM to 2 mM was sufficient in all instances to bring about a slow reduction in twitch potentiation to the control level. External Mg^{++}, 3 to 4 mM, reduced twitch tension to the control level in three to four minutes (fig. 1, panel 5).

Thus, ionic conditions that do not reduce transmitter release sufficiently to interfere with transmission can nevertheless abolish twitch potentiation.

Drug effects on the ACh receptor of the denervated diaphragm. Interactions of Ca^{++} and Mg^{++} with the facilitatory drugs in the innervated diaphragm suggest a presynaptic site for the process and are consistent with the concept of Werner (1960) that these drugs produce positive and negative after potentials in the motor nerve terminals in response to nerve stimulation, which modify the transmission process. Other workers have, however, emphasized the importance of end-plate sensitization (Katz and Thesleff, 1957; Karczmar *et al.*, 1965).

The ACh receptor of the denervated diaphragm offers a convenient approach to the postsynaptic receptor, which, as was mentioned in the introduction, is easy to study experimentally and has the further advantage that it is largely free of acetylcholinesterase. This means that changes in receptor sensitivity cannot be ascribed solely to anticholinesterase properties of the drugs.

Dose-response curves for the ACh-induced contraction of the denervated diaphragm were drawn as described previously (Freeman and Turner, 1969). The drug was then added to the organ bath and the ACh dose-response curve determined in the presence of the drug. Finally, the preparation was washed for greater than 30 minutes to remove the facilitatory drug, and the ACh contraction was checked to ensure that the sensitivity of the preparation had not altered. It was not always possible to carry out this

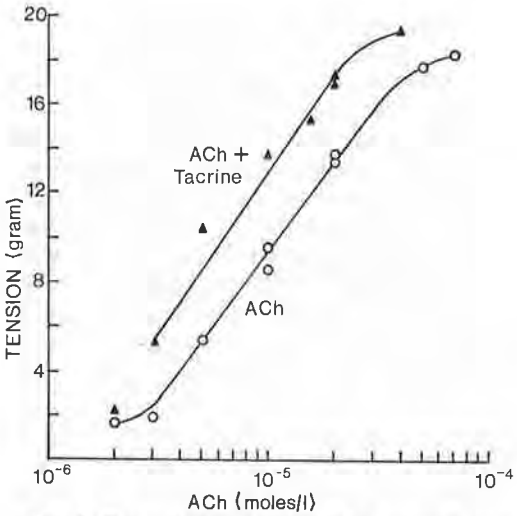

FIG. 3. Potentiation of the ACh-induced contraction of denervated rat diaphragm by tacrine (5×10^{-6} M). ACh (○——○); ACh + tacrine (▲——▲). Semilogarithmic scale. Each point refers to one observation. ACh response after wash out of tacrine was routinely checked at 10^{-5} M ACh.

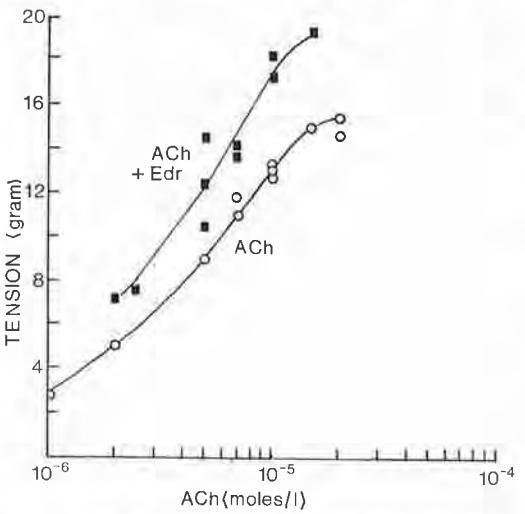

FIG. 4. Potentiation of the ACh-induced contraction of denervated rat diaphragm by edrophonium (5×10^{-6} M). ACh (○——○); ACh + edrophonium (■——■). Semilogarithmic scale. Each point refers to one observation. ACh response after edrophonium wash out was routinely checked at 10^{-5} M ACh.

final stage when tacrine was used, because of the slowness with which this drug was washed off the preparation. At least three levels of each drug were used in these experiments. Typical dose-response curves in the presence and absence of the facilitatory drugs are shown in figures 3 and 4. Data were not pooled for these figures, since the slope of the ACh dose-response curve tended to vary slightly among preparations (Freeman and Turner, 1969). Typical curves from groups of three diaphragms/drug are illustrated. It was noted that tacrine, edrophonium and 3-OHPEt$_2$MeA all potentiated the ACh-induced contraction at concentrations which potentiate twitch tension in the intact phrenic nerve-diaphragm preparation. The points about the potentiated ACh dose-response curves show more scatter than do control curves, and potentiation was consistently less at low levels of ACh than it was at levels producing nearly maximal contractions.

Higher levels (10^{-4} M) of edrophonium and 3-OHPEt$_2$MeA caused an initial potentiation of the ACh contraction which diminished with time of exposure to the drug until after one hour, when these drug levels had a depressant effect. Similar drug levels also brought about twitch potentiation followed by depression in the intact phrenic nerve-diaphragm preparation.

In contradistinction to these findings 3-OHPTEA caused a depression of the ACh-induced contraction at all levels tested (fig. 5). Depression of the ACh contraction at 10^{-3} M 3-OHPTEA was so intense that doses of ACh

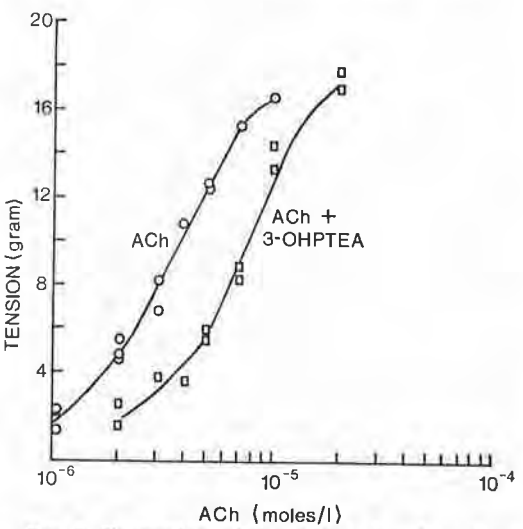

FIG. 5. Depression of the ACh-induced contraction of denervated rat diaphragm by 3-OHPTEA (10^{-4} M). ACh (○——○); ACh + 3-OHPTEA (□——□). Semilogarithmic scale. Each point refers to one observation. ACh response after wash out of 3-OHPTEA was routinely checked at 10^{-5} M ACh.

greater than 10^{-4} M were needed to elicit a contraction. Dose response curves at 10^{-4} M and 5×10^{-5} M 3-OHPTEA were parallel with the control, and the depression of the contraction could be entirely reversed by washing the preparation.

The effect of the facilitatory drugs on the depolarization of denervated muscle by ACh was also determined, since potentiation of the contraction could have been due to an effect on the process of excitation-contraction coupling.

The relationship between ACh concentration (10^{-6}–10^{-4} M) and depolarization was determined and was found to conform closely to that reported by Lüllmann and Reis (1967). Experiments were designed so that the membrane potentials of a small population of muscle cells were determined before addition of ACh to the organ bath. The normal value for membrane potential obtained from 37 sets of readings in denervated muscle was 67.8 ± 0.7 mV (S.E.M.). A series of six to eight penetrations was then made over a 4-minute period in the same area in the presence of ACh. After 20 minutes' wash the membrane potential had returned to the control level, and the readings taken were pooled with the first control group to obtain the ACh depolarization.

The effect of the facilitatory drugs on the ACh depolarization was determined at a fixed ACh concentration of 10^{-5} M. Groups of membrane potential measurements were made as before, and the effect of the facilitatory drug was estimated by subtracting the depolarization due to ACh in the presence of the drug from the potential measured in the presence of the facilitatory drug alone. The facilitatory drug concentrations were optimal as determined in the tension recording experiments and had no depolarizing effect on the membrane potential *per se*. The results of these experiments are shown in table 1; the drugs potentiated the ACh depolarization at the same concentration as they potentiated the ACh-induced contraction. The ACh depolarization was depressed by 3-OHPTEA (10^{-4} M), as would be expected from the preceeding data. This concentration of 3-OHPTEA did not affect the membrane potential *per se;* 3-OHPEt$_4$MeA (10^{-4} M) was also without effect. However, edrophonium (10^{-4} M) depolarized the membrane by 22 mV, and tacrine (5×10^{-5} M) caused a barely significant depolarization of 5 mV. These concentrations of edrophonium and tacrine caused a transitory potentiation of the indirectly elicited muscle twitch, followed by some depression.

TABLE 1

Facilitatory drug effects on the ACh depolarization of denervated muscle

Drug	ACh Depolarization		P
	Control	Treated	
	mV		
Edrophonium, 10^{-5} M	26.4 ± 1.9 (22)	35.9 ± 1.3 (22)	.001
3-OHPEt$_2$MeA, 10^{-5} M	23.3 ± 1.7 (15)	30.7 ± 1.4 (15)	.002
3-OHPTEA, 10^{-4} M	29.0 ± 1.6 (14)	14.8 ± 1.7 (15)	.001
Tacrine, 5×10^{-6} M	23.9 ± 1.8 (17)	33.8 ± 2.0 (15)	.001

Values shown are ± S.E.M.; the number of observations is shown in parentheses. Significance of difference of means was determined with Student's *t* test. ACh concentration throughout was 10^{-5} M. Muscle resting potential was not altered by these concentrations of facilitatory drugs.

It was noted that repeated exposure to ACh in the presence of a facilitatory drug over a period of several hours reduced the increase in depolarization. It is possible that repeated depolarization of the membrane for 4- to 5-minute periods may have led to a reduction in ionic gradients which were not adequately reconstituted *in vitro*.

Sensitivity of the ACh-induced contraction to divalent cation variation. It was shown previously (Freeman and Turner, 1969) that the ACh-induced contraction of the denervated diaphragm was insensitive to reduction in the level of external Ca^{++} or Mg^{++} to 0.15 mM or 0.1 mM, respectively. However, a 3-fold increase in the levels of these ions (4.5 mM or 3 mM, respectively) reduced the force of contraction which was also prolonged by the high Ca^{++} level.

The sensitivity of the potentiated ACh contraction to divalent cation variation was determined for all three potentiating drugs to obtain a comparison with the ionic effects on drug action in the intact phrenic nerve-diaphragm preparation. The results of these experiments are summarized in figures 6 and 7. As was noted previously, low Ca^{++} had little or no effect, whereas high Ca^{++} or Mg^{++} depressed the ACh contraction. Tacrine (5×10^{-6} M) brought about the usual potentiation of the contraction due to 10^{-5} M ACh; this potentiation proved to be insensitive to reduction in the external Ca^{++} or Mg^{++} level. Increased levels of these ions, how-

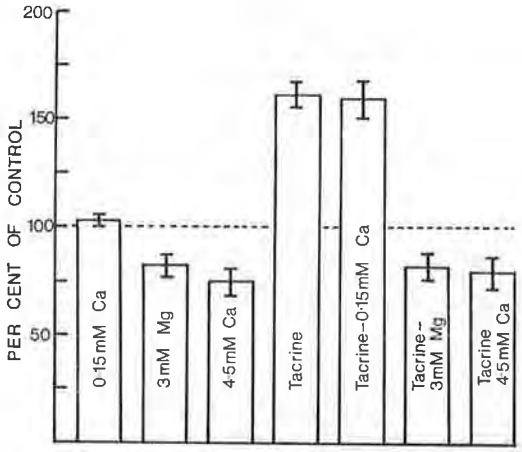

FIG. 6. The effect of variation in divalent cation level on ACh contraction of denervated diaphragm in the presence and absence of tacrine (5×10^{-6} M). The dotted line indicates the control contraction in normal bathing solution, ACh, 10^{-5} M. Vertical bars indicate ±1 S.E. of six or seven observations.

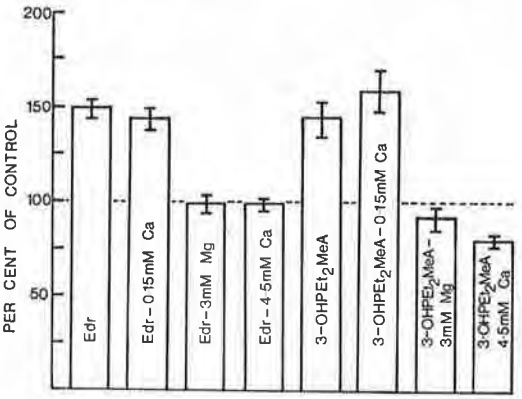

FIG. 7. The effect of divalent cation variation on the ACh contraction of denervated diaphragm in the presence of edrophonium or 3-OHPEt₂MeA (5×10^{-6} M). The dotted line indicates the control contraction in normal bathing solution, ACh, 10^{-5} M. Vertical bars indicate ±1 S.E. of six or seven observations.

ever, reduced the tacrine-potentiated contraction to rather less than the control value (fig. 6). These effects were essentially duplicated by edrophonium and 3-OHPEt₂MeA, (fig. 7).

It is of interest that the ionic effects on the drug-potentiated twitch of the intact phrenic nerve-diaphragm preparation are essentially opposite to those observed for the denervated diaphragm receptor. Thus low Ca^{++} abolishes twitch potentiation, but is without effect on the potentiated ACh contraction. Raised levels of Ca^{++} or Mg^{++} have opposing effects on the intact junction but both abolish potentiation in the denervated preparation.

Interactions with succinylcholine. Previous publications from this laboratory have emphasized the importance of presynaptic factors in SCh blockade of the rat neuromuscular junction (Freeman, 1968a,b; Freeman and Turner, 1968b). Drugs which reverse SCh blockade were shown to act largely by increasing the presynaptic output of ACh. Since the experiments reported in this study suggest that presynaptic factors are dominant in twitch potentiation of the intact diaphragm, it was of interest to investigate the ability of these drugs to relieve SCh blockade.

Tacrine was found to be devoid of any antidotal action against SCh blockade, although we confirmed the earlier observation that prior treatment with tacrine reduced the intensity of blockade (Ho and Freeman, 1965). This effect is opposite to that which would be expected if tacrine inhibition of pseudocholinesterase were a significant feature of the experimental situation. The three hydroxyanilinium compounds all had some antidotal action under our experimental conditions, although complete relief of SCh blockade was found only with 3-OHPEt₂·MeA (fig. 1, panel 7). 3-OHPTEA was less effective than the diethyl derivative and edrophonium brought about an average restoration of twitch tension of only 33% (see table 2). As was found previously (Freeman, 1968b) when considering antagonism of SCh blockade by pyridine-2-aldoxime methiodide, the antidotal action of the hydroxyanilinium compounds fell off sharply with the development of tachyphylaxis to

TABLE 2

Percent relief of SCh blockade by hydroxyanilinium compounds

Conc.	Edrophonium	3-OHPEt₂MeA	3-OHPTEA
M			
5×10^{-5}		93 ± 2 (5)	
10^{-4}	No relief	85 ± 5 (5)	No relief
5×10^{-4}	33 ± 3 (6)		45 ± 5 (5)
10^{-3}	Transient relief		47 ± 2 (5)

Values are ± S.E.M.; the number of observations is shown in parentheses. Concentration of SCh was 9×10^{-6} M.

SCh blockade. Thus, the figures shown in table 2 were obtained from first and second exposures to SCh, before the development of tachyphylaxis at or after the third exposure to SCh.

It was noted earlier in this paper that reduction of the external Ca^{++} concentration abolished twitch potentiation by the hydroxyanilinium compounds. It may be seen from figure 8 that antagonism of SCh blockade is also dependent upon the presence of a normal Ca^{++} level. SCh (9×10^{-6} M) and 3-OHPEt$_2$MeA (10^{-4} M) were added sequentially to a preparation in which twitch tension was slowly declining due to reduction in Ca^{++} level to 0.15 mM. SCh caused a temporary alleviation of the low Ca^{++} blockade, followed after one minute by a greatly increased rate of blockade. 3-OHPEt$_2$MeA added during the course of SCh-low Ca^{++} blockade did not affect the time course of the exponential decline in tension. However, when tension was reduced to zero, the Ca^{++} level was raised to 1.5 mM; this rapidly restored tension to 85% of the control level. Similar results were obtained with 3-OHPTEA and edrophonium, except that restitution of the external Ca^{++} level achieved only partial alleviation of the SCh blockade, as would be expected from table 2.

These results recall those found with the three isomers of pyridine aldoxime methiodide and emphasize the Ca^{++} dependence of the antagonistic action in both instances.

Although only the diethylmethyl hydroxyanilinium compound was found to be an effective SCh antagonist the converse applied in that SCh antagonized drug-induced facilitation extremely effectively. Figure 1, panels 1 and 3, shows the antagonism of facilitation due to tacrine and edrophonium by SCh at a concentration of 9×10^{-6} M. This concentration of SCh is the minimum dose necessary to produce complete blockade at an untreated diaphragm (Freeman, 1968a,b). At this concentration antagonism of twitch potentiation is followed by SCh blockade. It was found however that SCh, 9×10^{-7} M ($\times$ 0.1 the blocking dose), was sufficient to abolish twitch potentiation due to tacrine, edrophonium and 3-OHPTEA. However, 3×10^{-6} M SCh was necessary to abolish potentiation by 3-OHPEt$_2$MeA (fig. 1, panel 8). These findings are consistent with the observation of Standaert and Adams (1965) that post-tetanic repetition is depressed at SCh doses 10 times less than are required to depress transmission.

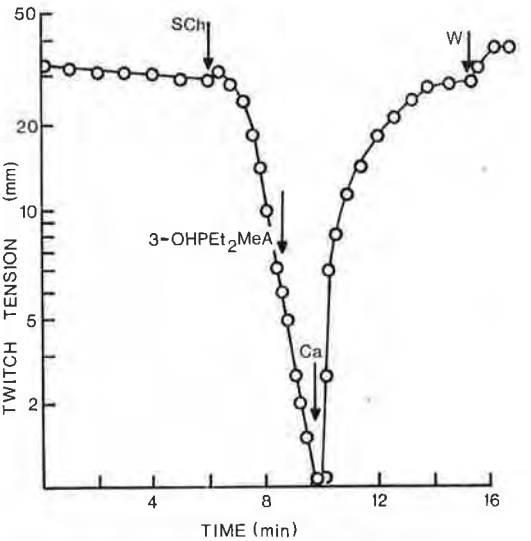

FIG. 8. The effect of SCh (9×10^{-6} M) and 3-OHPEt$_2$MeA (10^{-4} M) on partial blockade of the phrenic nerve-diaphragm preparation by 0.15 mM Ca^{++}. The drugs were added at the arrows. The external Ca^{++} level was restored to 1.5 mM at Ca. Time to half block ($T_{1/2}$) in low Ca^{++} = 24 minutes; after addition of SCh $T_{1/2}$ = 0.5 minute. Semilogarithmic scale; 25-mm twitch tension = 10 g.

DISCUSSION. Potentiation of the indirectly elicited isometric twitch may be due to facilitation of one or several steps in the process of neuromuscular transmission. Blaber and Karczmar (1967a) have discussed the likelihood of multiple cholinoceptive sites at the neuromuscular junction, all of which may interact with drugs producing facilitation. One may categorize these sites as follows: the motor end-plate, acetylcholinesterase and two sites at the nerve terminal, one of which may be the most distal node of Ranvier (Hubbard *et al.*, 1965; Freeman, 1968b).

Three of the four facilitatory compounds presently studied have been shown to potentiate both the ACh-induced contraction and the ACh-induced depolarization of the denervated diaphragm and consequently are likely to have a qualitatively similar effect on the postsynaptic membrane; the fourth compound (3-OHPTEA) had a depressant effect over a wide concentration range. All four facilitatory drugs are acetylcholinesterase inhibitors of various potencies (Ho and Freeman, 1965; Kuperman *et al.*, 1961).

It remains to discuss effects of these compounds upon the nerve terminals. Many authors have produced evidence of such effects (Standaert and Riker, 1967; Blaber and Karczmar, 1967a,b); however, neither the site nor the mechanism of these prejunctional effects has been determined.

The present evidence of the sensitivity of twitch potentiation to small changes in divalent cation concentration suggests that the unmyelinated terminals, which are the site of ACh release, undergo repetitive activity in the presence of these drugs either spontaneously or in response to stimulation (Werner, 1960). The possibility that this activity might originate in the most distal nodes of Ranvier, but be manifest in the terminal arborization, must, however, be considered. Thus, the Ca^{++}-independent antagonism of SCh blockade by tetraethylammonium and triethylcholine was previously ascribed to a nodal action of these drugs, wherein prolongation of the action potential invading the terminals increased the safety margin for ACh release in the presence of low Ca^{++} or SCh (Freeman, 1968b). The feasibility of such an effect may be inferred from the finding of Katz and Miledi (1967) that the release of ACh by brief depolarizing pulses applied to the terminals increases in more than linear proportion when the duration of the pulse is lengthened.

However, SCh antagonism by the hydroxyanilinium compounds is Ca^{++} dependent, which suggests that they act largely on the nerve terminals, where Ca^{++} plays a prime role in transmitter release (for references see Katz and Miledi, 1968). These authors obtained their evidence from amphibian preparations; however, it is likely to be valid also in mammalian preparations (Riker and Okamoto, 1969). One may further reason that SCh suppression of both drug-induced and post-tetanic potentiation is related to competition between this drug and Ca^{++} in the nerve terminal membrane, with consequent damping of potential oscillations (Freeman, 1968a,b).

Twitch potentiation cannot, however, be equated with the mechanism of SCh antagonism, as is clear from the present investigation and the work of other authors (Blaber and Karczmar 1967a,b). Although tacrine causes twitch potentiation, it shows no antagonism of SCh blockade. Further, it is an anticholinesterase of potency comparable to that of 3-OHPEt$_2$MeA and causes sensitization of the ACh receptor. Thus, properties other than these three must account for SCh antagonism by the hydroxyanilinium compounds.

The finding that facilitatory drugs all have some anticholinesterase activity may be regarded as a function of the structural similarity of all cholinoceptive sites rather than as a reflection of a causal relationship.

Structurally tacrine is unrelated to the hydroxyanilinium compounds. The amino group of tacrine is largely ionized at physiologic pH (pK_a = 9.95, Albert and Goldacre, 1946) but this compound lacks the substituted onium group that appears to be common to SCh antagonists (Freeman, 1968b). It is clear that grouping around the quaternary nitrogen is critical in this antagonistic action, since the diethylmethyl derivative is many times more active than either edrophonium or 3-OHPTEA.

In an evaluation of pre- and postsynaptic factors in drug-induced facilitation the weight of evidence in the present study falls in favor of a predominantly presynaptic action. Thus, the high temperature coefficient of facilitation is opposite in sign to the known increase in postsynaptic membrane sensitivity at low temperature. Further, the effects of variation in the divalent cation concentration of the bathing solution on facilitation in the intact junction are opposite to those found for the denervated receptor. Whereas the denervated diaphragm cannot be considered to offer more than a "reasonable" model of the postsynaptic receptor, the results here are so clear cut as to be unequivocal.

Conclusions. In the intact neuromuscular junction the four drugs studied (tetrahydro-4-aminoacridine, edrophonium, 3-hydroxyphenyldiethylmethylammonium and 3-hydroxyphenyltriethylammonium) caused twitch potentiation and spontaneous firing. Twitch potentiation was temperature sensitive, with an apparent energy of activation of approximately 26 kcal mol^{-1}. The potentiation was somewhat increased by 4.5 mM Ca^{++} ($\times$ 3 normal) but was abolished by 3 mM Mg^{++} ($\times$ 3 normal) or low Ca^{++} solutions.

The ACh-induced contraction of the denervated diaphragm was potentiated by all drugs except 3-hydroxyphenyltriethylammonium, which had depressant action over a wide concentration range. The ACh depolarization was

similarly affected. Potentiation of the ACh contraction of denervated diaphragm was insensitive to reduced levels of Ca^{++} or Mg^{++} but was reduced to the control levels or below by 4.5 mM Ca^{++} or 3 mM Mg^{++}. The divalent cation effects on the denervated receptor are essentially opposite to those on the intact junction.

Interactions with SCh are described. SCh abolished twitch potentiation of the intact preparation at low concentrations; only 3-hydroxyphenyldiethylmethylammonium proved to be an effective SCh antagonist. Structural groups around the quaternary nitrogen atom appear to be critical in this antagonistic action.

On the basis of the evidence presented it is suggested that drug-induced facilitation in the intact junction is largely a presynaptic action.

ACKNOWLEDGMENT. We wish to thank Mr. D. Amos for the synthesis of 3-hydroxyphenyldiethylmethylammonium and 3-hydroxyphenyltriethylammonium.

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