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.

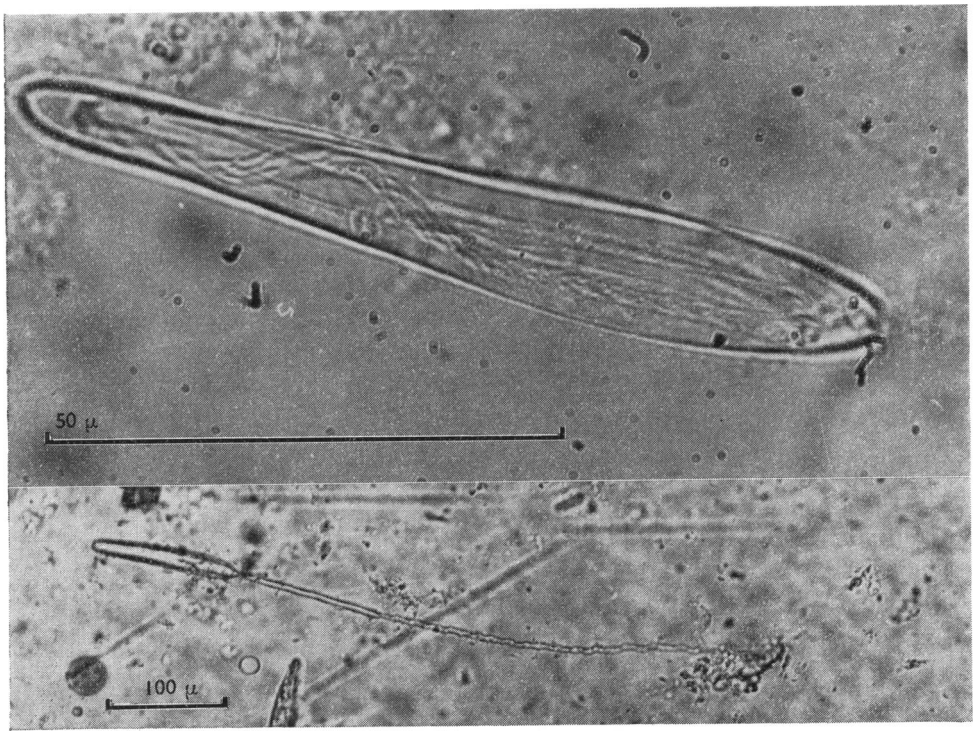

FIG. 1. Nematocysts isolated from frozen *Chironex* tentacle. These are the poison-containing microbasic mastigophore; tentacles contain several other types (Barnes, 1967). The upper picture shows the undischarged capsule; most were in this state in the frozen tentacle. The lower picture shows a discharged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

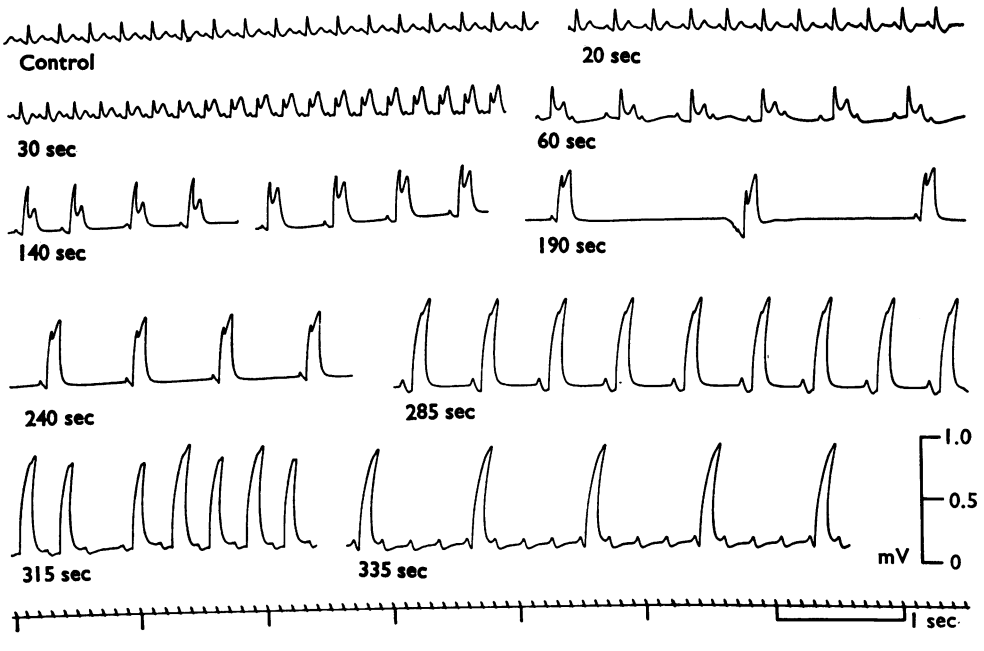

FIG. 2. Electrocardiogram changes following toxin injection. The first panel shows a segment of e.c.g. before injection, heart rate 260/min. There is an increased T-P interval and bradycardia 20 sec post-injection. T-wave enlargement is seen after 30 sec. At 60 sec there is 2:1 atrioventricular block. 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.

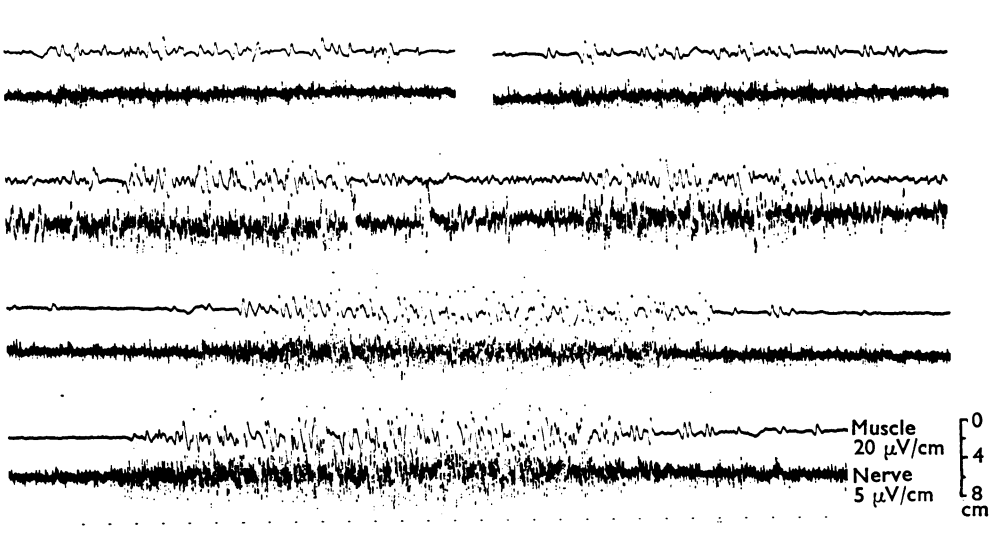

FIG. 3. Phrenic nerve and diaphragm activity following toxin injection. Upper trace shows record from myograph needle in the diaphragm, lower trace shows phrenic impulses. Panel 1 shows a control respiration. Panel 2 shows increased phrenic activity after a non-lethal toxin injection. Panel 3 shows irregular, 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)