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Abstract approved:

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There are well over 200 species of fishes which are known to be venomous, and which are capable of inflicting serious and potentially fatal wounds. Within this group, less than 5% have been studied even in the most preliminary manner and, to date, the basic chemical structure of even a single fish venom has not been elucidated. It is evident that the major reason for this apparent lack of knowledge is the inherent and extreme instability of fish venoms.

Lionfish (Pterois volitans) venom stabilization was achieved with the addition of protease inhibitors during venom apparatus homogenization, and was confirmed by assay on an isolated fish heart preparation.

Administration of stabilized lionfish venom to the isolated heart of the buffalo sculpin (Enophrys bison) resulted in a positive chronotropic effect from low doses (40 μg protein/ml), but produced a negative chronotropic effect from higher doses (100-200 μg protein/ml). Cardiac output changes coincided with

those of the heart rate, and no ionotropic changes were detected at any dose.

This same chronotropic effect difference produced by the higher doses of venom was also observed in the *in vivo* sculpin preparations. Venom administration to the live buffalo sculpin also produced a distinct increase in blood pressure not seen in the isolated heart preparations.

This apparent increase in peripheral resistance produced by lionfish venom administration was also confirmed by use of an isolated tail/intact vasculature preparation, and is assumed to be due to vasoconstriction.

Bioassay of the lionfish venom on juvenile buffalo sculpin resulted in a LD_{50} determination of 200 μg protein/kg.

Stabilized lionfish venom was also analyzed with gel electrophoretic and high performance liquid chromatographic (HPLC) techniques. Gel electrophoretic separation of the venom revealed high molecular weight components in the range of 29,000 to 116,000. HPLC analysis with a Waters SW-300 protein column revealed three peaks when measured at 219 nm. Reverse phase HPLC analysis offered a greater resolution of the venom components and resulted in approximately 20 peaks. Fractions eluted from this separation which were administered to buffalo sculpin failed to produce any observable adverse effects.

The varied effects produced by lionfish venom cannot be explained by a single mechanism. Although this venom may have direct effects on the cardiovascular system, indirect effects

caused by the release of natural pharmacological substances and neurogenic reflex mechanisms must also be considered.

Chemical Stabilization and Pharmacological Characterization of the Venom of the Lionfish, *Pterois volitans*

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PREFACE

The venomous property of certain fishes has been recognized for thousands of years, however, scientific investigations have only begun recently on these fishes and their venoms. The study of Fish Venomology encompasses many fields such as anatomy, physiology, pharmacology, and biochemistry.

This work represents a study of the venom of a common tropical marine teleost fish which, in defense, is capable of inflicting extremely painful and occasionally serious wounds in humans. The emphasis of this project was to first develop an effective method of stabilizing the venom from natural inactivation or degradation; and secondly to characterize its pharmacological and physiological effects on a non-mammalian species, specifically another marine teleost. This is based on the premise here being that the effects of a fish venom may be more pronounced when introduced into other fishes, the animals against which the venom is presumably used in defense.

The techniques developed and described in this work, especially that of the chemical stabilization of the venom with the use of protease inhibitors, can hopefully be used in the further study of this and of other fish venoms.

GENERAL INFORMATION

Lionfishes belong to the family Scorpaenidae, the scorpionfishes, and are widely distributed throughout the tropical and temperate seas of the world. They are found abundantly in the Indo-Pacific, the near-shore areas of Australia, Japan, and China, in the Indian Ocean, and the Red Sea (Allen and Eschmeyer, 1973; Halstead, 1978; Southcott, 1970, 1977).

Lionfishes of the *Pterois* genus, of which there are six species, are commonly known by a variety names such as lionfish, zebrafish, tigerfish, scorpionfish, turkeyfish, featherfish, firefish, stingfish, butterfly fish, lalong, and many more. These fish are commonly found in shallow water associated with rocks or reefs, and are easily recognized by their large plume-like pectoral fins (Atz, 1962; Halstead, 1978).

Bottard (1889) apparently was the first to report the existence of a venom apparatus in *Pterois* sp. (Halstead *et al*., 1955). The venom of these fishes is contained in the associated tissues of the dorsal, pelvic, and anal spines. The largest plume-like pectoral fins contain no venom-producing tissues. Envenomation occurs through mechanical pressure on the spine, which generally ruptures the integumentary sheath. The venom is thus emptied mechanically and diffuses into the wound produced by the fin spine (Russell and Brodie, 1974).

Stings from lionfish cause extreme pain, local swelling, and discoloration of the skin. In severe cases, lionfish stings have caused extreme hypotension, and cardiovascular collapse (Saunders

and Lifton, 1960). There have been unconfirmed reports of deaths caused by lionfish stings, however, in all of the published clinical accounts of non-fatal lionfish envenomations, no individual has had more than three, of the eighteen total, venomous spines puncture the skin. Thus, lionfish do indeed pose a potential hazard to swimmers, divers, fishermen, and hobbyists (Steinitz, 1959; Ray and Coates, 1958).

CHEMICAL STABILIZATION AND PHARMACOLOGICAL CHARACTERIZATION OF THE VENOM OF THE LIONFISH, *PTEROIS VOLITANS*

INTRODUCTION AND LITERATURE REVIEW

There are approximately 1000 species of fishes which are known to be either poisonous or venomous (Russell, 1969). Although these terms "poisonous" and "venomous" have often been used synonymously in the literature, there is an important distinction between them.

POISONOUS fishes are those fishes which cause a biotoxication upon ingestion of their tissues which are either partially or entirely toxic (Russell and Brodie, 1974). Halstead (1970, 1978) has further classified this group of fishes into: those which contain a poison within their musculature, viscera, or skin (Ichthyosarcotoxic), within their gonads (Ichthyootoxic), or within their blood (Ichthyohemotoxic). Halstead also adds a fourth division to include those fishes which produce a toxin by glandular secretion, but which lack a true inflicting apparatus (Ichthyocrinotoxic). Poisonous fishes and their associated toxins have been studied in great detail in the past few decades, and the chemistry and pharmacology of these toxins have been extensively reviewed (Russell, 1965, 1967a,b, 1969; Halstead, 1967, 1978). In some cases, the study of poisonous fishes has led to the discovery of valuable pharmacological and neurological tools, such as tetrodotoxin from the puffer fishes (family Tetraodontidae) and the porcupine fishes (family Diodontidae) (Kao, 1964, 1966, 1972).

VENOMOUS fishes, on the other hand, are those fishes which are capable of producing a toxin in a highly developed secretory organ, gland, or specialized group of cells, and which can administer this venom with some form of spine or stinging apparatus (Russell, 1969; Russell and Brodie, 1974; Halstead, 1970, 1978). Venomous fishes and their toxins, however, have not been studied in much detail. Halstead (1978) estimates that out of the entire group of venomous fishes, of which there are over 200 species, less than five percent of these have been studied even in a cursory manner. In fact, the basic chemical structure for even a single fish venom has never been determined.

The study of the biological activities of fish venoms is complicated by a number of factors which also help to explain the lack of basic knowledge in this field. The primary reason is that a characteristic common to all fish venoms is their extreme instability. Most fish venoms lose activity at room temperature and some are labile even at temperatures of 0°C (Halstead, 1978). Activity can also be lost or significantly decreased even upon lyophilization (Russell, 1965, 1967a, 1969). Fish venom research is further complicated by the fact that quantitative and qualitative differences in venom composition may exist intraspecifically as well as interspecifically. A fish venom can possibly vary within the individual animal at different times of the year or under varying environmental conditions (Russell, 1971).

The toxins of venomous fishes differ considerably in their pharmacological and chemical properties from the toxins of the

poisonous fishes, and especially from the toxins of other aquatic and terrestrial venomous animals (Russell, 1969). For example, there is no relationship between lionfish venom and puffer fish poison.

Although these inherent unstable properties of fish venoms have impeded much progress in this field, a number of chemical, pharmacological, and anatomical studies on a wide variety of venomous fishes were done in the late 1950's and early 1960's.

For the purposes of this thesis, only a few of the most important and relevant works in fish, and specifically scorpaenid, venomology addressing the chemical and pharmacological studies will be reviewed.

For an extensive review of all of the research done in the field of Fish Venomology, the reader is referred to the works of Halstead (1959, 1965, 1967, 1970, 1971, 1978, 1980) and the reviews by Russell (1965, 1967a,b, 1969, 1971). These reviews also cover the numerous other groups of venomous fishes such as sharks, chimeras, stingrays, toadfishes, weeverfishes, catfishes, surgeonfishes, etc., not discussed here, but which are as equally important, dangerous, and relatively unstudied (see Birkhead, 1967; Cadzow, 1960; Cain, 1983; Carpenter, 1965; Castex, 1967; Colby, 1943; Collette, 1966; Cross, 1976; Datta et al., 1982; Evans, 1907, 1920, 1921, 1923, 1943, 1944, 1945; Halstead and Mitchell, 1963; Herre, 1949; Minton, 1974; Mullanney, 1970; Pacy, 1966; Roche, 1973; Scoggin, 1975; Thulesius et al., 1983).

SCORPIONFISHES

There are approximately 80 species of fishes belonging to the suborder Scorpaenoidei (families Scorpaenidae and Synancejidae) which have been determined to be venomous (Nelson, 1984; Halstead, 1978). This group encompasses the true scorpionfishes (genus *Scorpaena*), the lionfishes or turkeyfishes (genus *Pterois*), and the stonefishes (genus *Synanceja*). As stated previously, studies on fish venoms have been hampered due to the instability of these venoms. However, certain chemical similarities of the venoms of the scorpionfishes (suborder Scorpaenoidei) have been reported, and data obtained on the venom of one species has been correlated with results from venom studies of other species (Halstead, 1970, 1978; Russell, 1965, 1967a, 1967b, 1969; Saunders, 1959a, 1959b, 1960).

According to Halstead (1970, 1978) the study of scorpaenid venomology dates back to the time of Aristotle, however, no significant chemical, pharmacological, or physiological studies were reported until the late nineteenth century.

Bottard (1889) reported some of the first morphological descriptions of the venom apparati of scorpionfishes, and he was the first scientist to perform any chemical and pharmacological studies with the venoms of these fishes. He aspirated the fluid from the dorsal fin spine sacs of the stonefish (*Synanceja horrida*) and found this crude venom extract to be a clear, blue color with cellular elements present. This crude venom caused paralysis and death when injected into frogs. The venom also caused extreme

pain and paresthesia when he injected a drop into his own leg. Bottard apparently was also the first to describe the existence of a venom apparatus in the lionfish, *Pterois volitans* (Halstead *et al.*, 1955).

More detailed studies on the chemistry, pharmacology, and physiological effects of scorpionfish, and other venomous fish, venoms are of a much more recent origin. In the separate laboratories of Russell, Saunders, Taylor, Weiner, Austin, and others, attempts were made at extracting, stabilizing, purifying, and characterizing the various components of the Scorpionfishes; stonefishes, true scorpionfishes, and lionfishes (Austin *et al.*, 1961, 1965; Carlson, 1972; Carlson *et al.*, 1970, 1971, 1973; Coates *et al.*, 1980; Duhig, 1928; Duhig and Jones, 1928; Russell and Brodie, 1974; Saunders, 1958, 1959a,b,c, 1960; Taylor, 1963; Weiner, 1958, 1959a,b).

Russell worked extensively in these same areas with weeverfish and stingrays, as did Skeie with weeverfish (Rodrigues, 1972; Russell, 1953, 1961; Russell and Bohr, 1962; Russell and Lewis, 1956; Russell and Van Harreveld, 1954, 1956; Russell *et al.*, 1957, 1958; Skeie, 1962a,b,c; Smith *et al.*, 1978).

Halstead, Cameron and Endean, and others, during this time worked on the anatomic and morphological descriptions of the venom apparati of these fishes (Cameron and Endean, 1966, 1970, 1971, 1972, 1973; Cameron and Lewis, 1982; Cameron *et al.*, 1981; Endean, 1961; Halstead, 1956, 1957; Halstead and Bunker, 1953; Halstead and Dalgleish, 1967; Halstead and Modglin, 1950; Halstead and

Smith, 1954; Halstead *et al.*, 1953, 1955a,b,c, 1956, 1971, 1972; Roche and Halstead, 1972).

1) *Stonefishes* (genus *Synanceja*)

Weiner (1958, 1959a, 1959b) found the venom from the dorsal spines of *Synanceja trachynis* (same species as *Synanceja horrida*) to be opalescent with a pH of about 6.0. He found his extracts were most stable at a pH range of 7.0 to 7.6, and were unstable at or below pH 4.0, and at or above pH 8.6. Lyophilized venom extracts, stored in a desiccator for several months at 4°C, showed no loss of activity. Freezing and thawing, however, did cause an activity loss. Venom extracts which were heated at 50°C for five minutes lost all lethal activity. Weiner found his extracts to be proteinaceous and of high molecular weight.

Weiner studied some of the pharmacological effects of stonefish venom (1958, 1959a). He found the LD_{50} of the venom in mice to be about 200 μg protein/kg body weight (i.p.). Hemodynamic changes were observed when the venom was injected into mice, guinea pigs, dogs, and fowl, and reactions were dose and route dependent. Weiner suggested that the hypotensive effect of this venom could be due to an arteriolar dilation causing a lowering of peripheral resistance, however, he did not rule out the possibility of a direct effect of the venom on the myocardium. Weiner (1959b) discovered the stonefish venom to be antigenic in mice, rabbits, and horses, and was able to produce an antivenin (Commonwealth Serum Laboratories, Melbourne, Australia) for clinical use

against stonefish envenomations which has been used successfully in clinical trials (Phelps, 1960; Matic-Piantanida *et al*., 1979).

According to Saunders (1959c), the lethal components of stonefish (*Synanceja verrucosa*) venom are closely associated with non-dialyzable, high molecular weight proteins. Deakins and Saunders (1967) were able to achieve a ten-fold chemical purification of *Synanceja horrida* venom using starch gel electrophoresis. Saunders and Tokes (1961) found that lyophilized and glycerol-treated extracts retained 50 to 100% of their toxicity after one year of storage at -20°C.

Saunders (1958, 1959c, 1960) has described some of the effects of stonefish (*Synanceja horrida* and *S. verrucosa*) venom in mice and rabbits. He found low doses to cause arterial hypotension and respiratory changes, and larger doses caused electrocardiographic (ECG) alterations, respiratory arrest and death. The author concluded that the primary action of the venom was on the cardiovascular system which, in the higher doses, was associated with myocardial damage. Saunders found the i.v. LD_{50} of this venom in mice to be 200 μg protein/kg body weight. They also estimated that the venom extracted from the spines of one fish would contain 10,000-25,000 LD_{50}'s for mice.

Austin and his co-workers (1965) performed chemical studies on the venom of the stonefishes *Synanceja trachynis* and *S. verrucosa*, and found both to have similar elution profiles when chromatographed on Sephadex G-75. In both cases, the lethal activity was only associated with the first peak of increased

absorbance at 280 nm, representing a high molecular weight protein which was excluded from the gel matrix. These workers separated the S. trachynis venom with the cationic exchanger carboxymethylcellulose and discovered the lethal component was associated with the last of the three protein peaks resolved. Estimating molecular weight by ultracentrifugation, these researchers were able to determine that the toxic component of both stonefish venoms had two fractions with molecular weights of approximately 15,000 and 150,000. The authors attempted to stabilize the lethal fractions using thiol reducing and oxidizing agents, BAL, hydrogen bond breaking agents, versene, and a variety of buffers over a wide range of pH values, all without success.

Austin et al. (1961) reported somewhat different pharmacological results than did Saunders (1958, 1959c, 1960). The authors found that Synanceja horrida venom is a non-specific myotoxin, having a direct paralyzing effect on skeletal, cardiac, and smooth muscle. The venom was found to produce a transient vasoconstriction in the isolated rabbit hind limb preparation. In the Langendorff isolated heart preparation, a low dose (30 μg) of venom caused an increase in coronary flow, but no change in heart rate. Higher doses resulted in a bradycardia, a negative ionotropic effect, and a reduction in coronary flow. The venom sometimes caused an A-V block, and the authors concluded that the venom has a direct toxic action on the heart and interferes with conduction.

According to Austin et al. (1965), there are four major components in stonefish venom: 1) hyaluronidase, 2) a capillary

permeability factor, 3) a cardiovascular active factor which produces a dose-dependent hypotension, and 4) a pain producing factor.

Stonefishes possess the most lethal, painful, and dangerous venom of all of the venomous fishes, and envenomations from them usually requires immediate intensive care.

2) True Scorpionfishes (genus *Scorpaena*)

Studies on the effects of the venom of a *Scorpaena* species was begun by Pohl (1893) who worked with the venom of *Scorpaena porcus*, and also with the venom of a weeverfish, *Trachinus draco* (as cited by Halstead, 1978). Pohl determined that the venoms from both species had similar cardio-inhibitory effects on frogs, however, the *Scorpaena* venom was less potent and more variable in its effects. Dunbar-Brunton (1896), also working with *Scorpaena* venom, found that sub-cutaneous injections of the venom into guinea-pigs produced evidence of pain, motor paralysis of the limbs, convulsions, respiratory distress and death (Halstead, 1978). These preliminary results with *Scorpaena* venom were confirmed independently by Phisalix (1931), and De Marco (1937).

More recently, Taylor (1963), Carlson *et al*. (1970, 1971, 1973), Carlson (1972), and Schaeffer *et al*. (1970, 1971), have worked extensively with the venom of the California scorpionfish, *Scorpaena guttata*.

Taylor (1963) found that extracts from the spines of *Scorpaena guttata* maintained at 40°C for five minutes lost 60

percent of their lethal activity, whereas extracts stored at 4°C retained 75 percent of their lethal activity for up to 24 hours. Taylor found the extracts to be extremely labile in a variety of chemical reagents. He was unsuccessful in stabilizing the venom extracts in 5.0 mM reduced glutathione (GSH) alone, or in 1.0 mM ethylene diaminotetraacetic acid (EDTA) alone, and 1.0 mM parachloromercuribenzoate (PCMB) completely inactivated the lethal activity of the extracts when stored for one hour at 5°C. However, Taylor found that 1.0 mM diisopropylfluorophosphate (DSFP), a protease inhibitor, had a very slight stabilizing effect. Taylor concluded that fresh S. guttata spines yielded much more potent venom extracts than those which had been kept frozen for longer than 24 hours, and that repeated freezing and thawing inactivated the lethal activity.

Taylor chemically fractionated S. guttata venom extracts on a Sephadex G-75 column (fractionation range 3,000-80,000 molecular weight) and was able to recover 53 percent of the original lethality. The first peak, a high molecular weight protein excluded from the matrix of the gel, contained the lethal fraction. He separated the venom with a DEAE-cellulose column, but was unable to obtain a pure lethal fraction. The lethal fractions from the DEAE-cellulose separation were then separated electrophoretically and the lethal proteins were estimated to have molecular weights between 850,000 and 1,300,000. Dialysis of the crude venom resulted in no lethal activity being detected outside of the membrane.

Taylor observed some of the physiological effects of the California scorpionfish venom on a variety of test animals. He observed that the manifestations of toxicity in anesthetized rabbits were essentially identical to reports from other scorpaenid venoms (acute hypotension and respiratory distress with low doses, and myocardial injury and respiratory arrest with high doses). Taylor found the effects of the venom to be route dependent, and venom administered intraperitoneally or subcutaneously had no lethal effect even at higher doses, as compared to the venom administered intravenously. The author found the venom to have little effect when injected into an octopus, however, it produced alterations in respiration and locomotion, and death when injected into a tidepool sculpin, Clinocottus analis australis.

Carlson (1972) compared the lethality of Scorpaena guttata venom obtained by two different methods of extraction (Schaeffer et al., 1971). Their "aspiration" method involved stripping the integumentary sheath from each spine and then aspirating the tissue from the anterolateral glandular grooves with a micropipette connected to a flask and vacuum source. The supernatants after centrifugation were lyophilized and stored at 5°C. Their "batch" method involved immersing a number of stripped spines into cold distilled water (Russell and Brodie, 1974, contradict their own procedure, however, by stating that under no circumstances should venomous spines be washed with or exposed to fresh water) for five minutes (3 washings) and the decanted solution lyophilized and stored at 5°C.

Carlson (1972) found his "aspiration" method to give a relatively purer (LD_{50} in mice = 1.0 mg protein/kg body weight i.v.), albeit less stable, product than his "batch" method (LD_{50} in mice = 2.6 mg protein/kg). The author found no significant differences in the lethality of the venom extracts which were prepared immediately from sacrificed fish, or from specimens which had been stored on ice for up to 12 hours. Carlson assayed his venom extracts on rats, crabs, rabbits, cats, and dogs, and found that rats were equally sensitive to the venom as the mice. Shore crabs (*Pachygrapsus crassipes*) were not affected by the venom even at doses of 40 mg dried crude venom/kg. Rabbits were the most sensitive animal (LD_{50} = 0.3 mg protein/kg body weight), whereas the dogs and cats required considerably larger venom doses to produce death.

Carlson (1972) studied the effects of a number of physical parameters and chemical reagents on the *S. guttata* venom stability. The author found a loss of toxicity in lyophilized samples, as compared with fresh samples, even when stored at -60°C. Carlson's extracts were most stable in a pH range of 7.4-8.0, and were the most unstable at lower pH values. Samples exposed to heat (50°C and 80°C) lost their lethal activity, and samples stored at 5°C remained relatively stable for up to 72 hours. Carlson tested the effects of GSH, cysteine, and PCMB on the lethal activity of his venom extracts, and found these reagents provided *no* stabilization. In the case of the PCMB, $1x10^{-2}$ M and $1x10^{-4}$ M concentrations enhanced the activity loss. Carlson did

find, however, that Cleland's reagent afforded some stability to the lethal property of the venom for up to 21 days.

Crude S. guttata venom was chromatographed by the author on a Sephadex G-50 column (fractionation range 1,500-30,000 molecular weight) and three peaks were obtained. The first peak was the only one possessing lethal activity and was lethal to mice at a dose of 1.5 mg protein/kg body weight. When the crude venom was chromatographed on a Sephadex G-200 column (fractionation range 5,000-600,000 molecular weight), it was again resolved into three peaks. The second of these peaks (representing 20% of the total protein) contained the lethal fraction which was lethal to mice at a dose of 0.9 mg protein/kg. This represented a three-fold increase in lethality over the starting crude venom, and it was the first report of a fraction of a fish venom that was more lethal than the crude material (Carlson, 1972; Russell and Brodie, 1974). Taylor (1963) estimated, by DEAE cellulose separation and subsequent electrophoresis, the lethal protein(s) of Scorpaena guttata venom to have a molecular weight between 850,000 and 1,300,000. However, Carlson, Schaeffer, Russell and others in that laboratory estimated by column chromatography that the lethal property of the venom is associated with protein(s) having a molecular weight of more than 50,000 and less than 800,000 (Russell and Brodie, 1974).

Carlson (1972) performed quite an extensive array of pharmacological, and in particular cardiovascular, studies in rats, cats, rabbits and dogs. In the isolated rat atria preparation,

S. guttata venom produced a primary, dose-related, muscarinic effect, and also secondary beta adrenergic stimulation. Carlson states that the primary effect is most likely due to the release of endogenous acetylcholine, causing the observed negative ionotropic and chronotropic effects. Carlson was able to confirm this with pre-treatment of the rat atria with atropine and/or hemicholium. Carlson was also able to confirm the secondary positive response by inhibiting the venom effect with both a beta adrenergic blocking agent, and with the depletor reserpine.

In anesthetized cats, scorpionfish venom produced marked changes in the arterial blood pressure, central venous pressure, heart rate, electrocardiogram (ECG) and ventilation. An intravenous administration of 0.9 mg protein/kg was fatal in six of fifteen cats tested. However, repeated intravenous doses of 0.09 mg protein/kg injected at 30 minute intervals produced no lethal or cumulative effect. The 0.9 mg protein/kg dose produced a profound drop in systemic arterial pressure, which was followed by a period of hypertension in the non-fatal cases. Heart rate changes included a sinus tachycardia which always occurred and was sometimes followed by a sinus bradycardia, or arrhythmias. ECG alterations included inverted T waves, ST segment changes, premature atrial and ventricular contractions, and bundle branch block. Larger doses (2.0-2.5 mg protein/kg i.v.) caused cardiac standstill and respiratory arrest within 30 seconds.

Carlson found very similar cardiovascular effects from scorpionfish venom administration in anesthetized rabbits, although

with much lower doses (0.1 mg protein/kg i.v.), and also in anesthetized and conscious dogs at higher doses (0.5 mg protein/kg i.v.). From these experiments, it is evident that Scorpaena guttata venom has a marked effect on the cardiovascular system, and Carlson suggests that these effects are probably due to a sudden change in the circulating blood volume. Carlson states that part of the hemodynamic responses to the venom involve cholinergic mechanisms and that atropine inhibits the initial decline in arterial pressure and the ECG changes. The most dramatic cardiovascular effect of this venom is the consistent precipitous fall in systemic arterial pressure. The authors working with this scorpionfish venom concluded that the mechanism of action is not entirely a direct one, but one that involves cholinergic mechanisms, autopharmacological substances, and neurogenic reflexes (Carlson, 1972; Carlson et al., 1971; Russell and Brodie, 1974).

Additionally, it was determined that commercially available antisera for stonefish (Synanceja sp.) envenomations does not offer any protection against scorpionfish (Scorpaena guttata) venom (Carlson, 1972).

Envenomations from the California scorpionfish produce immediate and intense pain at the area of injury, and may spread throughout the effected extremity in minutes. The wound usually becomes swollen and ischemic, and discoloration of the skin is not uncommon. Halstead (1951) describes a detailed clinical account of a Scorpaena guttata envenomation, and states that serious or

deleterious stings from this fish occur with fishermen frequently, although no fatalities have ever been reported.

3) Lionfishes (genus *Pterois*)

In comparison to the amount of research done with stonefish (genus *Synanceja*) and scorpionfish (genus *Scorpaena*) venoms described above, relatively little work has been done with lionfish venom.

Bottard (1889) was the first to report the existence of a venom apparatus in the lionfish, and he concluded that this venom apparatus of *Pterois* sp. was identical to what he had found in the scorpionfish, *Scorpaena* sp. (Halstead *et al*., 1955). Halstead *et al* (1955) described, in detail, the complete anatomy of the venom apparatus of the lionfish, *Pterois volitans*.

No significant pharmacological or physiological studies had ever been attempted with lionfishes until the studies with *Pterois volitans* in 1959 (Saunders and Taylor, 1959; Saunders, 1959a). They homogenized the venomous spines of their specimens in a 0.9% NaCl solution and found the resultant crude venom extracts to be a turbid reddish-orange solution with a pH of 7.0. The crude extracts were bioassayed in mice within one hour of venom preparation and then attempted to preserve the remaining venom extracts by either lyophilization or by the addition of 40% glycerol, and storage at -20°C. The venom preparation and initial bioassays were performed "in the field" and consequently Saunders and Taylor were unable to perform a protein analysis on the fresh

extracts, and thus quantitative doses were not reported. The authors state that an i.v. injection of the fresh venom extracts killed the mice in about 30 seconds to 30 minutes depending on the relative dose, and the symptoms produced were: ataxia, partial or complete paralysis of the legs, and muscular weakness. They reported that with the fresh extracts, the amount of venom in one specimen of lionfish contains 2500 LD_{50} doses for mice.

Subsequent bioassays performed with the reconstituted lyophilized, and glycerol-treated venom extracts indicated a mean LD_{50} in mice of 1.1 mg protein/kg body weight. Saunders and Taylor estimated that these stored extracts lost an average of 30% of their activity. However, these workers report that their storage techniques allowed retention of 40 to 90% of the venoms original activity. They explain this discrepancy by stating that precise determination of stability was not possible due to the limited number of mice used (n=15).

Saunders and Taylor tested the pharmacological effects of their stored venom extracts on the anesthetized rabbits, and found the effects produced by both types of stored venom to be identical. Low doses produced a slight fall in blood pressure and an increase in respiratory rate, with no effect on heart rate or ECG. Large doses caused a more pronounced drop in blood pressure and increase in respiratory rate, and evidence of myocardial injury (flattening of T wave, displacement of the S-T segment), or conduction defects such as bundle branch block. This large dose did not cause a change in heart rate. Fatal doses (mean LD_{50} in

rabbits = 200μg protein/kg i.v.) produced the same symptoms initially, along with other ECG changes such as extrasystoles, bundle branch block, ventricular tachycardia, and ventricular fibrillation, and finally death.

The authors concluded that the primary action of the venom is on the cardiovascular system, specifically producing a marked hypotension. They believe the actions of the lionfish venom to be very similar to the effects of the venoms of the stonefishes *Synanceja verrucosa* and *S. horrida*. They add that the active substance(s) in *Pterois volitans* venom are non-dialyzable and are identical with or are closely associated with protein.

No other study to date has been done on the venom of any species of lionfish.

Stings by lionfishes cause immediate intense and sometimes burning pain which quickly radiates from the site of injury to other parts of the body. Lionfish envenomations seem to cause more severe pain and are more dangerous than those from stingrays, scorpionfishes, and weeverfishes, however, they are not as dangerous as the stings from the stonefishes (Russell, 1965). Deaths have been reported (Halstead, 1978), although not confirmed, from lionfish stings, and in some cases the non-fatal envenomations can be very serious (Ray and Coates, 1958; Steinitz, 1959; Saunders and Lifton, 1960).

From the above review of the current literature in Fish Venomology, and specifically from the obvious lack of knowledge

about the lionfishes and their venom, the goals of this project were:

1) To devise a simple method of venom extraction and preparation.

2) To stabilize, chemically or otherwise, the lionfish venom extracts from degradation.

3) To determine some basic biochemical knowledge about the venom.

4) To pharmacologically characterize the effects of lionfish venom on the cardiovascular system, by means of live animal and isolated organ preparations, of another marine teleost.

MATERIALS AND METHODS

LIONFISH COLLECTION AND MAINTENANCE

Lionfish (*Pterois volitans* L.), which were captured alive and imported from the Philippine Islands, were obtained commercially (The Blue Heron Fishery, Tukwila, WA.). Upon arrival, the fish were maintained at 25°C in 125 gallon closed-system, artificial seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio) aquaria (34°/oo). The fish were fed live shrimp twice weekly, and were held for at least one month prior to venom extraction.

VENOM EXTRACTION AND PREPARATION

Three methods of venom extraction, which did not involve the killing of the lionfish were studied. The rubber sheet method of Endean (1961) and Cameron and Endean (1966), the sponge method of Carlilse (1962), and the hypodermic needle method of Saunders and Tokes (1961) were all attempted unsuccessfully. The details of these methods and their results with the lionfish are discussed in the appendix.

Due to the structural morphology of the lionfish venom apparatus, and specifically the relationship of the venom glands to the structure of the spines (Fig. 1), the aspiration and batch methods of Schaeffer (1972) and Schaeffer *et al*. (1971) (see Introduction) were deemed unfeasible. The method of venom extraction used with success, was a modification of one developed by Saunders and Taylor (1959), who used lionfish.

The venom apparatus of the lionfish consists of the 13 dorsal, three anal, and two pelvic spines, their associated venom glands lying within the anterolateral grooves (Figs. 1 & 2), and the enveloping integumentary sheaths (Halstead et al., 1955).

In an attempt to account for possible differences in crude venom due to animal size, age, sex, etc., three to six lionfish were used in any one venom preparation.

Lionfish were decapitated and their entire venom apparatus cut away at the base of the spines and placed on ice. All subsequent steps of the venom preparation were conducted at 0-4°C. The tissue was minced with scissors, weighed, and transferred, in approximately one gram portions, to clean polypropylene tubes. The tissue was homogenized using a polytron (Brinkmann Instruments) at a setting of six in a 0.9% NaCl solution (3.0 ml/g tissue), or in one of two chemical cocktails being tested for stabilizing effects (see Methods section 3). The homogenates were then centrifuged at 1000 x g for 10 minutes. The resulting supernatants were decanted and either immediately assayed on the isolated heart preparation, or lyophilized and stored at -85°C.

STABILIZATION STUDIES

To minimize the possibility that proteolytic degradation was responsible for the instability of the lionfish venom, two methods of chemical protease inhibition were studied and assayed on the isolated buffalo sculpin heart (see Methods section 4a).

Figure 1. Schematic drawing showing a cross-sectional view of a typical dorsal spine of the lionfish, Pterois volitans.

Key:
- A - dorsal spine
- B - venom glands lying within the anterolateral grooves of the spines
- C - surrounding integumentary sheath

(modified from Halstead, 1970 and 1978)

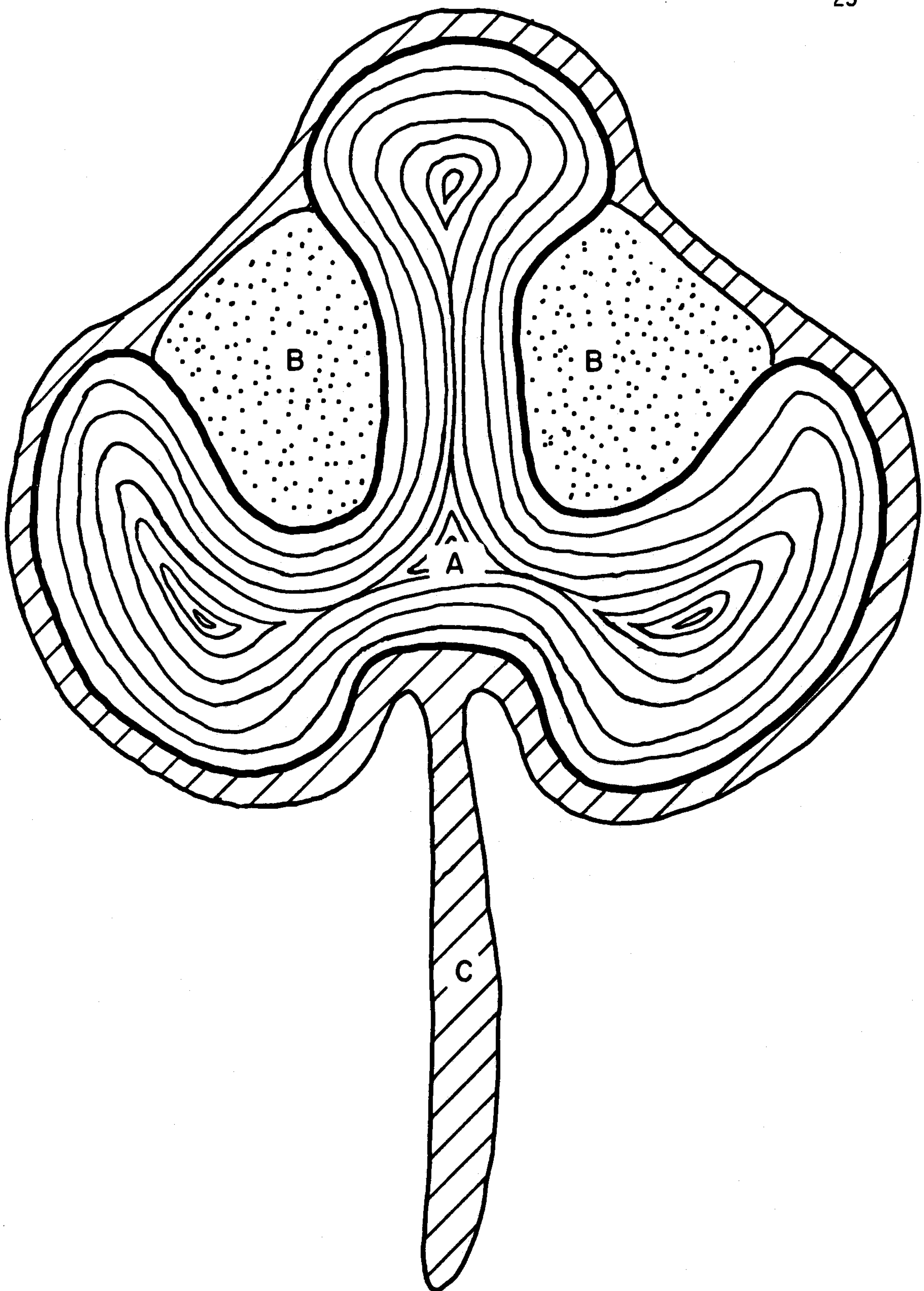


Figure 1

Figure 2. Drawing of a typical dorsal spine of the lionfish, *Pterois volitans*, showing the location of the anterolateral-glandular grooves.

(modified from Halstead, 1970 and 1978)

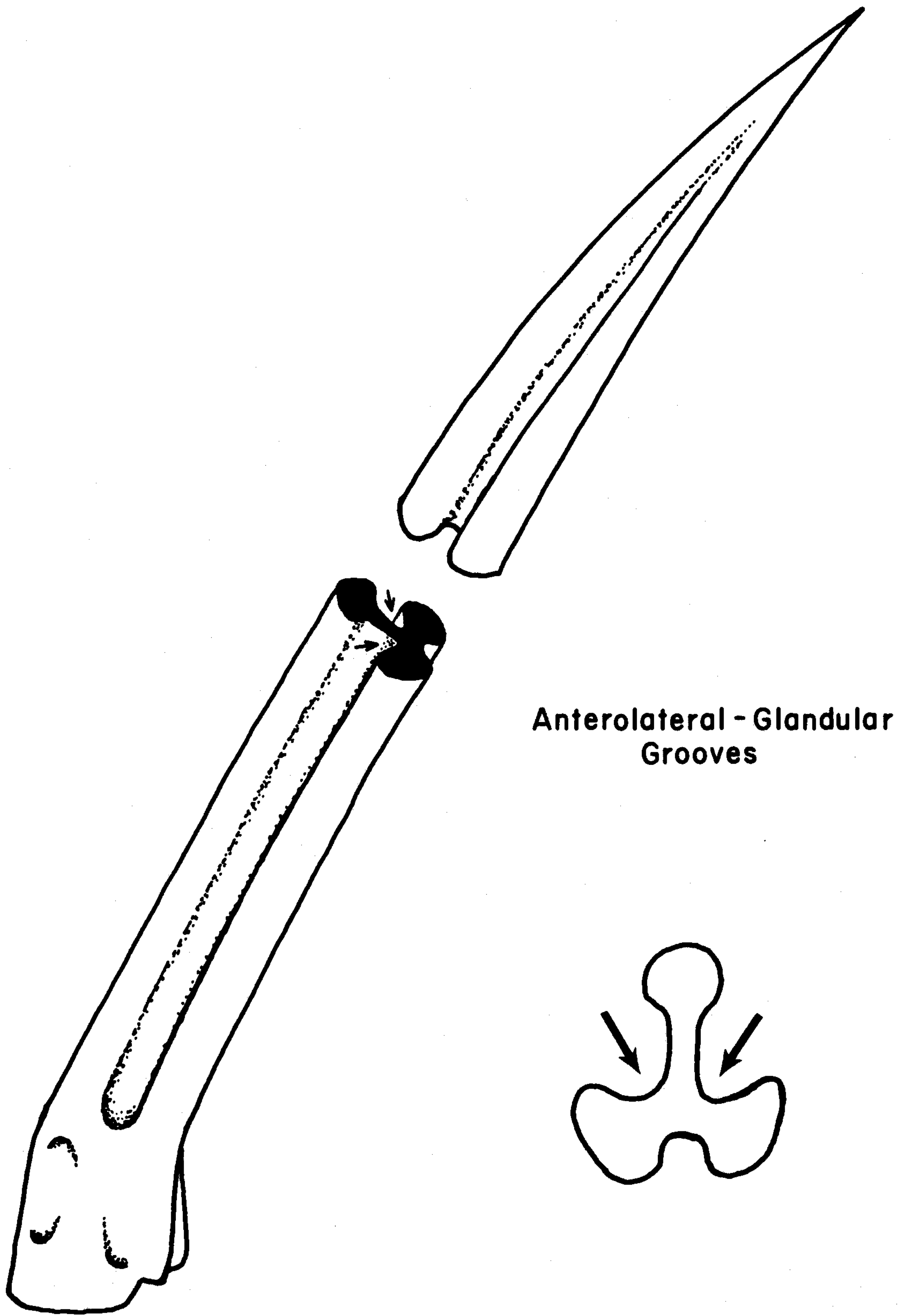


Figure 2

1) Protease Inhibitor Cocktail (PIC)

Protease inhibitors active against each of the four known catalytic classes of proteases (Barrett, 1977; Hartley, 1960) were added to the excised, minced venom apparati just prior to homogenization (3.0 ml buffered PIC per gram of tissue). Soybean trypsin inhibitor (10 μg/ml) was used to inhibit serine and thiol proteases, EDTA (1 mM) was used to chelate the metalloproteases, pepstatin A (5 μg/ml) was used to inhibit the carboxypeptidases, and bacitracin (1.0 μg/ml) was added to reduce bacterial contamination. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

2) α_2-Macroglobulin

α_2-macroglobulin has been reported to be a universal protease inhibitor, and can bind, trap, or inactivate nearly all endoproteases (Barrett, 1981; Barrett and Starkey, 1973; Barrett *et al.*, 1974; Starkey and Barrett, 1977; Harpel, 1975, 1976). A buffered solution of human plasma α_2-macroglobulin (1.0 μg/ml) (Sigma) was added to the minced lionfish venom apparati just prior to homogenization (3.0 ml/g tissue), to test its stabilizing effects on the lionfish crude venom extracts.

PHARMACOLOGICAL STUDIES

1) In Vitro Isolated Heart Experiments

Buffalo sculpin, *Enophrys bison* (Girard), were used as the experimental animals due to their local abundance and easy maintenance. Adult specimens, ranging from 300 to 1000 grams,

were collected from Yaquina Bay, Newport, Oregon by otter trawl. The sculpin were held indoors in 50 gallon aquaria with aerated, filtered, and U. V. light-treated running seawater, maintained at 12°C. Sculpins were fed a marine animal gelatin diet (see Appendix) twice weekly.

The animals were killed by a blow to head and then positioned on their dorsal side to allow a midventral incision to be made which exposed the heart chamber. The pericardial sac was opened and the hearts were excised so as to include the bulbous arteriosus, and as much of the diffuse sinus venosus as possible. Excised hearts were immediately placed in a cold (0-4°C) marine teleost saline (sculpin saline, Stuart _et al_., 1983) and flushed to remove residual blood and clots. Isolated hearts were first weighed, and then ligated to heat-molded, flair-tipped cannulae (PE 90) at the bulbous arteriosus and at the sinus venosus (see Fig. 3). Hearts, and their attached cannulae, were then mounted to a modified Langendorff perfusion apparatus (Langendorff, 1895; Gunn, 1913; Bennion, 1968; Stuart _et al_., 1983) by the sinus venosus cannula (Fig. 4). The bulbous arteriosus cannula was attached to the central port of a pressure transducer (Gould-Statham P23ID). A 15 cm length of glass tubing (4 mm inside diameter) was mounted vertically to the exhaust port of the transducer to create a resting back pressure of 15 cm H_2O (1.5 kPa) for the hearts to work against (Turner and Driedzic, 1980) (Fig. 3). The hearts were perfused through the chambers with 12°C sculpin saline while submerged in an oxygenated saline bath also maintained at 12°C

Figure 3. Schematic diagram showing the attachment of the isolated buffalo sculpin heart to the constant perfusion pressure manifold and to the pressure measurement transducer. Note that the transducer is kept at the same level as the heart, and the resting pressure column exerting a back pressure on the heart.

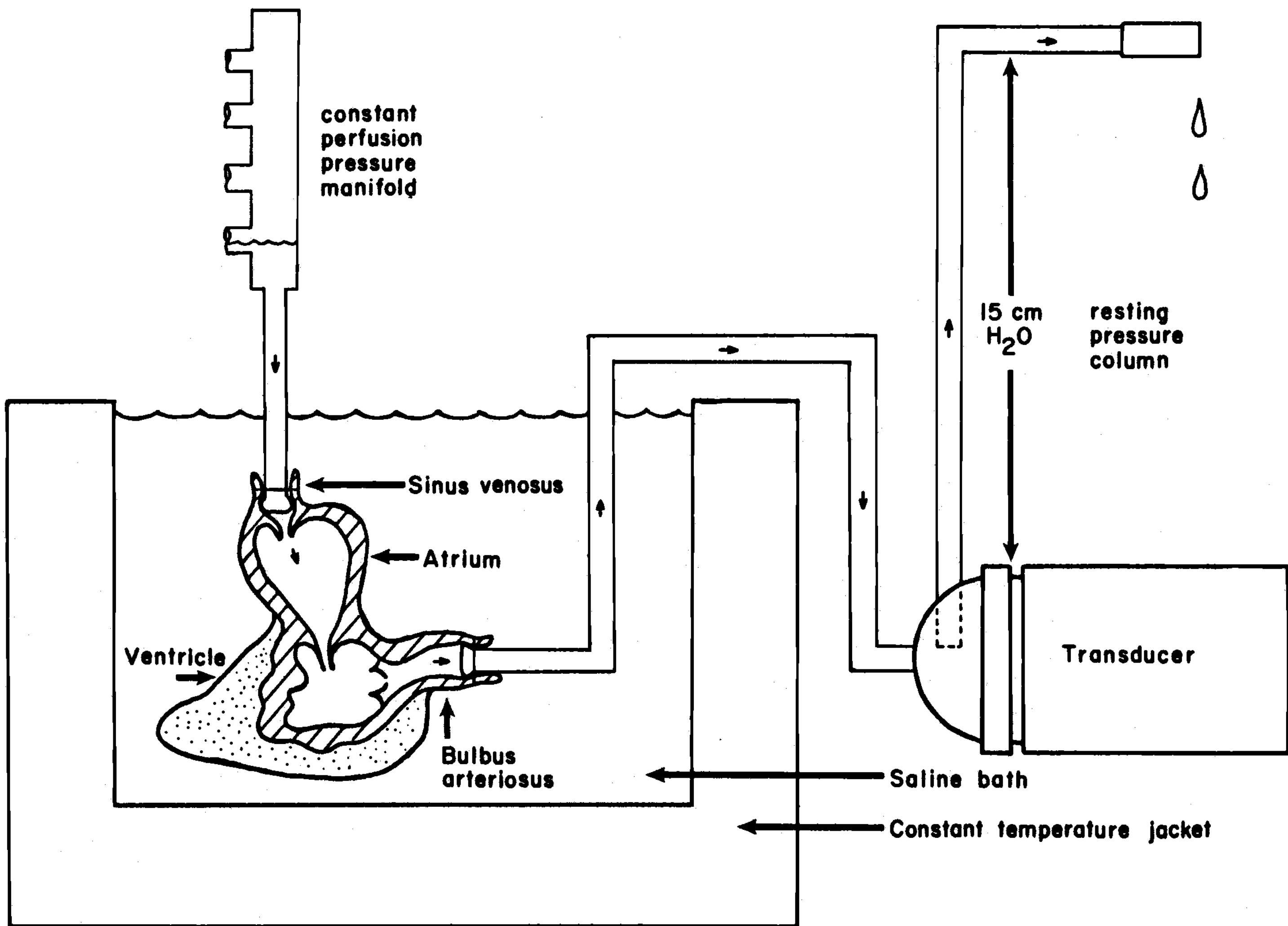


Figure 3

Figure 4. Schematic diagram showing a simplified overview of the modified Langendorff apparatus.

Key:
- A - sculpin saline reservoir
- B - alternate reservoir for saline + drug perfusion solutions
- C - cooling columns (12°C)
- D - in-line stopcock for bolus administrations of venom
- E - refrigerated recirculating water bath for maintaining perfusate and saline bath at 12°C
- F - chart recorder
- G - constant perfusion pressure manifold
- H - isolated sculpin heart in saline bath
- I - water jacket
- J - pressure transducer
- K - resting pressure column

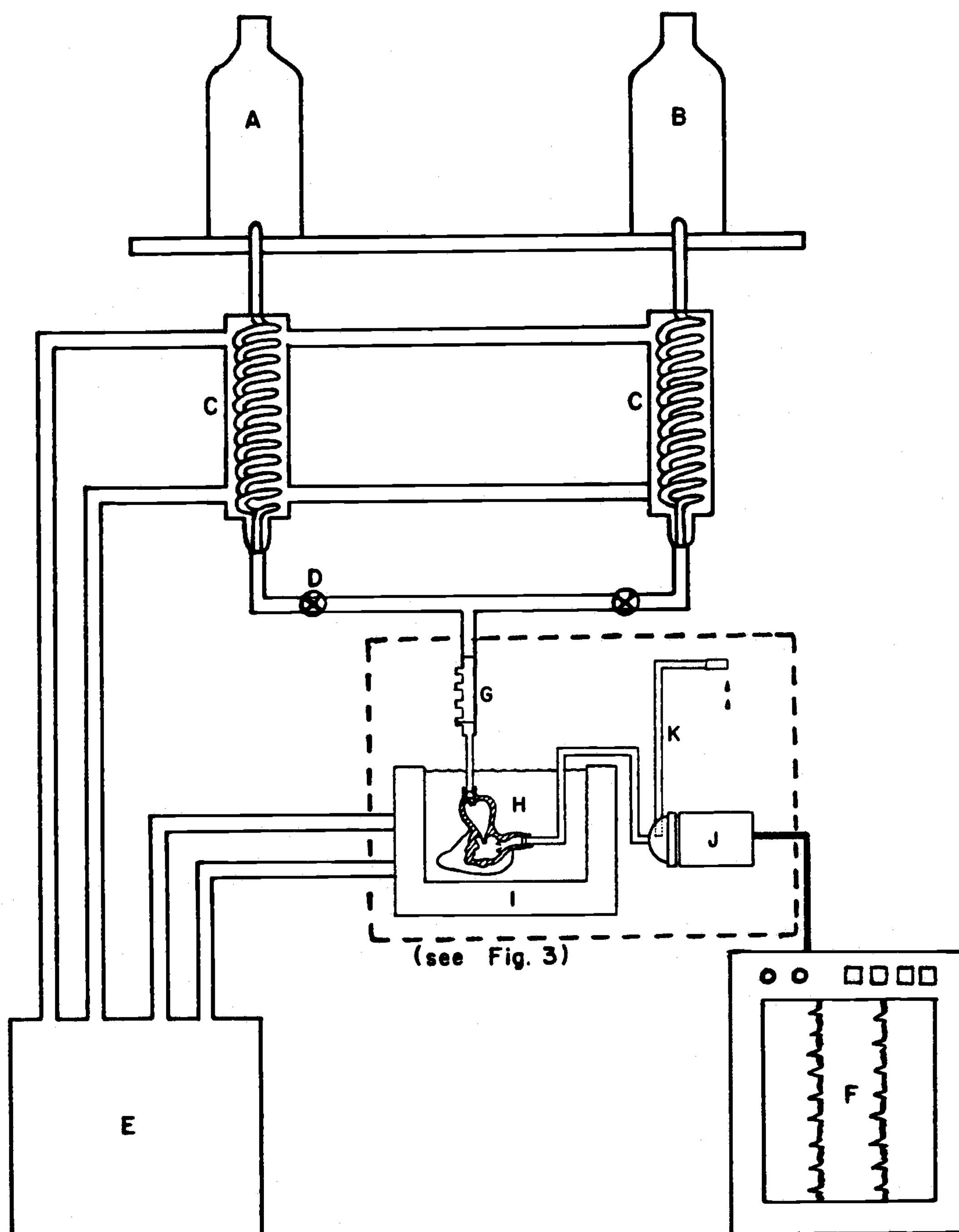


Figure 4

(Neslab RTE-8 refrigerated circulating bath) (Fig. 4). Heart rate and pulse pressure were measured with the pressure transducer which was connected to a transducer amplifier (Gould 11-4307-04) and recorded with a Gould-Clevite Brush Mark 220 chart recorder. Cardiac output was determined by collecting the perfusate, exhausting from the resting pressure column, in a graduated cylinder over a known time interval.

Hearts were allowed to stabilize for 30 to 45 minutes before the administration of a drug or the venom. In all experiments, the venom was introduced in a low volume bolus, via a stopcock attached to the saline perfusate supply line (Fig. 4). A minimum of three hearts were used in each test, and each heart served as its own control. Statistical analyses were by one-way analysis, or by Student's t-test.

2) In Vivo Cardiovascular Experiments

Buffalo sculpins (E. bison) weighing between 250 and 500 grams were anesthetized in an isolated seawater tank containing 70 mg/l tricaine methanesulfonate (MS 222; Sigma). Upon losing their righting ability, animals were transferred to a surgical table which allowed for the continuous irrigation of the fish's gills with an anesthetic-seawater solution, or normal seawater (Smith and Bell, 1967).

The branchial arteries of the second or third gill arch were cannulated with a 50 cm length of polyethylene tubing (PE 50; Intramedic) containing a small volume (0.2 ml) of 500 I. U. of heparinized (Sigma) sculpin saline (Fig. 5). The cannulae were

Figure 5. Schematic drawing representing the surgical vascular cannulation of a buffalo sculpin via the branchial arteries of the gills. Upper right schematic also shows close relationship of the heart, aortas, and gills.

Key: A - dosing syringe
B - three-way stopcock
C - 23 gauge hypodermic needle
D - polyethylene cannulae (PE 50)
E - physiological pressure transducer
F - surgical hemostats used to hold gill cover open during surgery

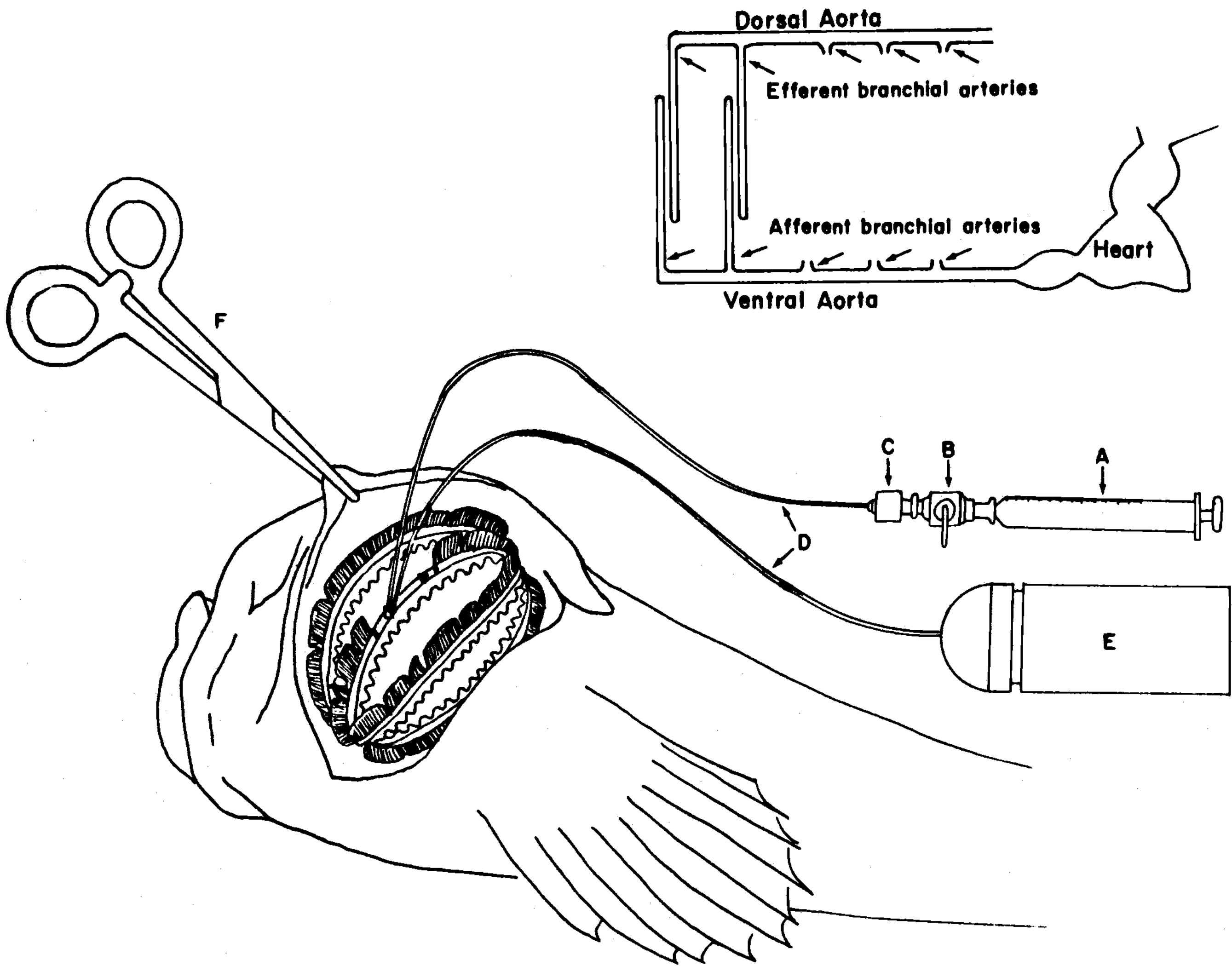


Figure 5

sutured into place along the gill arches and also to the skin of the fish. This cannulation procedure is described in detail in the appendix.

Two cannulae were usually implanted into each fish. One cannula was used for the administration of a drug or the venom by means of a three-way stopcock and a syringe (Fig. 6). The other cannula was used to simultaneously monitor heart rate and blood pressure via a pressure transducer (Gould-Statham P23De) interfaced with a transducer amplifier (Gould 11-4307-04) and a chart recorder (Gould-Clevite Brush Mark 260).

The cannulated fish were secured in plexiglass restraining cages and returned to their anesthetic-free, running seawater aquaria to recover. Recordings of heart rate and blood pressure were begun immediately, however, drugs or venom were not administered for 24 hours. Norepinephrine (10 μg/kg) and/or acetylcholine (2.0 μg/kg) (Sigma) were used as control agents before the administration of venom. Control animals were injected with a similar volume of sculpin saline.

3) In Vitro Isolated Tail / Intact Vasculature Experiments

Buffalo sculpins (*E. bison*) weighing between 200 and 400 grams were killed by a blow to the head, and the entire tail area was immediately cut away just proximal to the anus (vent). The isolated sculpin tails were weighed and placed in a cold (0-4°C) tray and kept moist with sculpin saline. Both the dorsal aorta and the caudal vein were cannulated with polyethylene tubing (PE 50, 90, or 100 depending on tail size and vessel diameters).

Figure 6. Schematic drawing representing a cannulated buffalo sculpin, in the plexiglass restraining cage, ready for pharmacological testing. Note that the pressure transducer (H) is at the level of the fish's heart for accurate recording.

Key:
- A - dosing syringe
- B - three-way stopcock
- C - 23 gauge hypodermic needle
- D - indwelling cannula in branchial artery for the direct i.v. administration of saline, drugs, or venom
- E - indwelling cannula in branchial artery for the monitoring of ventral aortic blood pressure and heart rate
- F - the experimental animal, buffalo sculpin (*Enophrys bison*)
- G - plexiglass restraining cage
- H - physiological pressure transducer (attached to amplifier and chart recorder)
- I - ring stand to hold transducer at the level of the fish heart
- J - constant temperature (12°C) running seawater aquarium

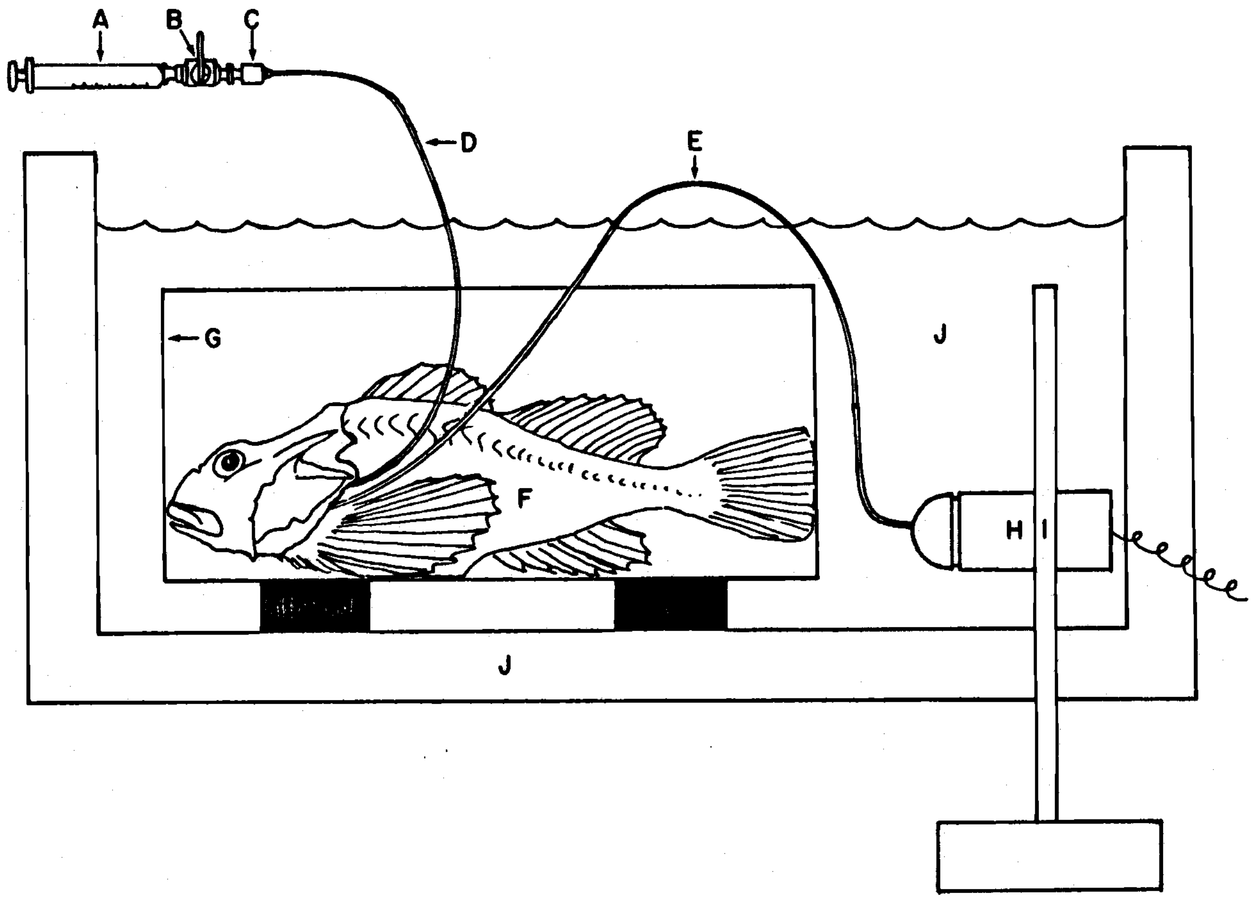


Figure 6

Surgical silk (4-0, Ethicon) was tied around the trunks of the isolated tails to prevent leakage and ensure a tight cannulae fit.

The dorsal aorta (inflow) cannulae were 25 cm in length and were connected to a leur-locking fluid manifold (Perfectum). A 60 cc syringe was connected to the opposite end of the manifold to infuse sculpin saline under a constant flow via a syringe pump (Sage Instruments, 341 A). To the two side ports of the manifold were attached: a pressure transducer (Gould-Statham P23ID) connected to a chart recorder (Gould-Clevite Brush Mark 220), and a dosing syringe for the administration of drugs or venom (Fig. 7). Norepinephrine (10 μg/kg) and acetylcholine (2 μg/kg) were used as the control agents before the administration of other drugs or the venom.

Due to the preliminary nature of this preparation, only qualitative results were obtained.

4) Bioassay

A sequential method of bioassay was performed using juvenile (100 to 300 grams) buffalo sculpin (*E. bison*). A multiple sample modification of the "up-and-down" method for bioassay (Dixon and Mood, 1948; Brownlee *et al.*, 1953) developed by Hsi (1969) was used. The bioassay was carried out sequentially: animals were treated in sequence, and the dose level used for each animal was dependent upon the response of the previous test. This method has been estimated to need fewer than two-thirds the number of animals conventionally used in bioassays, yet still yielding

Figure 7. Schematic drawing representing the isolated sculpin tail/intact vasculature preparation.

Key: A - constant pressure syringe pump
B - 60 cc saline reservoir syringe
C - pressure monitoring manifold
D - dosing syringe
E - physiological pressure transducer
F - inflow cannula
G - outflow cannula
H - dorsal aorta
I - caudal vein
J - isolated buffalo sculpin tail

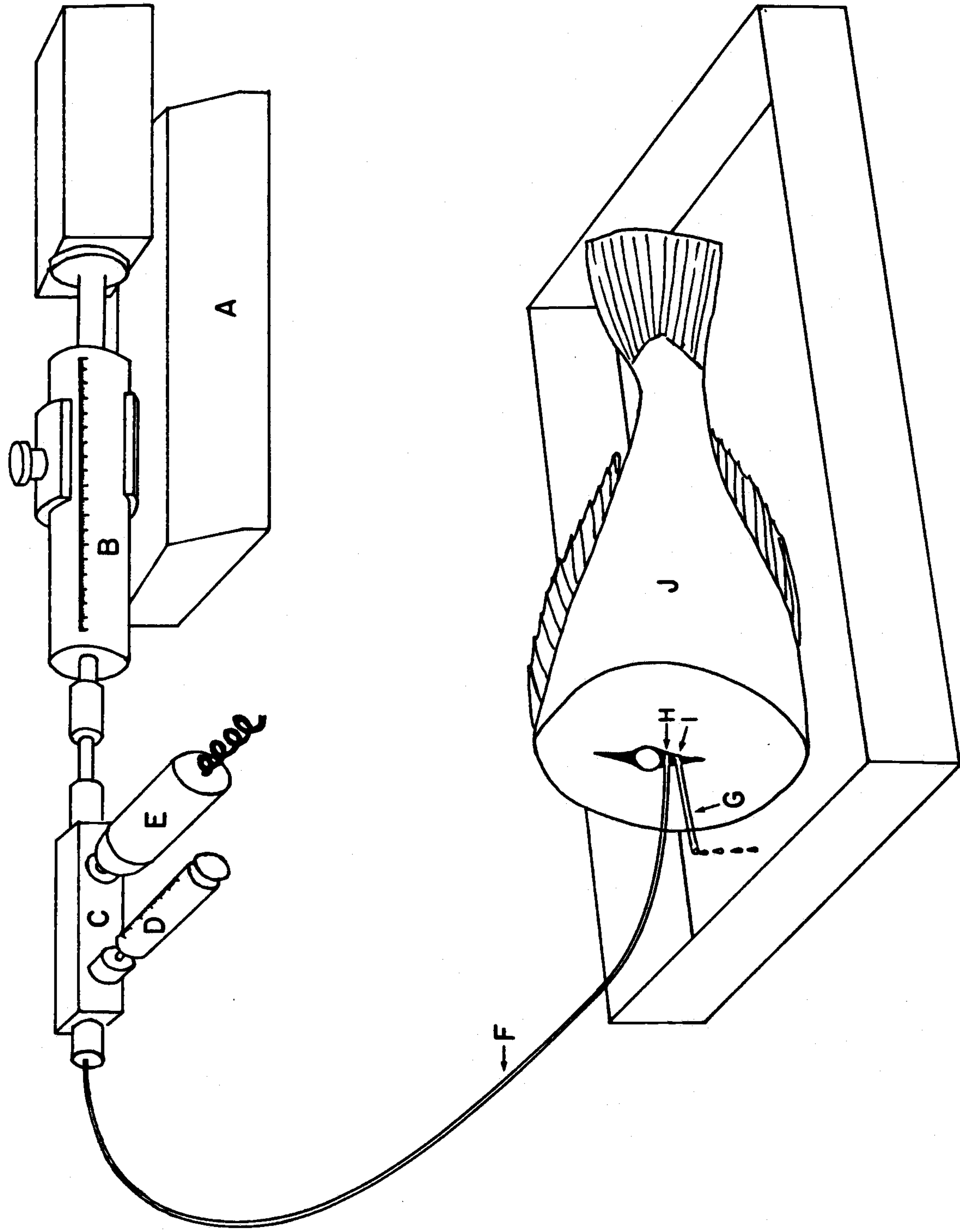


Figure 7

the same degree of precision (Brownlee et al., 1953; Chanter and Heywood, 1982).

Sculpins were administered crude venom, reconstituted from the lyophilized state, which was prepared with the protease inhibitor cocktail. Venom was administered via a syringe by cardiac puncture.

5) Liver Function Experiment

The effect of lionfish venom on the liver function of two specimens of buffalo sculpin (*E. bison*) was determined by measuring the elimination of the organic anion sulfobromopthalein (BSP; Sigma) from the blood plasma. The clearance rate of BSP has been extensively used to evaluate liver function in man, laboratory mammals, and recently in fishes, because of its selective hepatic uptake and biliary excretion (Weber et al., 1979). The ability of the liver to remove this dye from the blood is an indicator of the liver's ability to remove other substances that are normally removed by biliary excretion (Harper, 1973).

Two sculpin (444g and 566g) were surgically cannulated (see Methods section 4b and Appendix) to allow access to the vascular system for the purposes of BSP administration and continuous blood sampling.

Control liver function was assessed by following the disappearance of BSP from the plasma after a bolus administration of 15 mg BSP/kg, and repeated 20 μl blood sampling. Perturbed liver function was assessed by following the disappearance of BSP

from the plasma after bolus administrations of 15 mg/kg BSP, and 200 mg protein/ml of reconstituted crude lionfish venom.

The BSP levels in the 20 µl blood plasma samples were estimated by a modification of the method of Seligson et al. (1959).

PROTEIN DETERMINATION

A modification of the Bradford dye-binding assay was used to determine the concentration of protein in each batch of the venom extracts (Peterson, 1983). A calibration curve was prepared for each determination using crystalline bovine serum albumin (Sigma). The optical density was read at 595 nm using a Gilford 2200 spectrophotometer.

SDS GEL ELECTROPHORESIS

Samples were prepared and analyzed by sodium dodecyl sulfate (SDS) polyacrylamide (12.5%) electrophoresis according to the procedure of Laemmli (1970). 1.5 mm thick, vertical slab gels were polymerized with 0.48% TEMED (N,N,N',N'-Tetraethyethylenediamine) and 0.52% ammonium persulfate. The gels were stained with 25% isopropanol, 10% acetic acid, and 0.25% Serva Blue R dye. The SDS molecular weight markers used were: myosin MW=205,000; E. coli β-galactosidase MW=116,000; phosphorylase B MW=97,400; bovine plasma albumin MW=66,000; egg albumin MW=45,000; carbonic

anhydrase MW=29,000; trypsinogen MW=24,000; soybean trypsin inhibitor MW=20,100; and α-lactalbumin MW=14,200.

All chemicals were obtained from Sigma.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC mapping of crude lionfish venom extracts was performed analytically and preparatively using an Altex 420 microprocessing HPLC system with a Synchropak reverse-phase column (10 cm x 4.1 mm I.D.) (Syn Chrom, Linden, IN). The buffers were 0.1% (v/v) trifluoroacetic acid (TFA; Eastman Kodak, Rochester, NY), and 0.1% TFA in acetonitrile (CH_3CN; HPLC grade, J.T. Baker, Phillipsburg, NJ). Peptides were eluted using a 0-60% (v/v) gradient of CH_3CN in 30 minutes. The flow rate was 1.0 ml/minute. Peptides were monitored at 219 nm with a Hitachi model 100-40 spectrophotometer equipped with a flow-through cell. The elution profiles were recorded (Perkin Elmer model 56 chart recorder) and 1.0 ml fractions were collected (Gilson microfractionator) throughout the gradient.

Two venom samples were analyzed using gel sieving HPLC with a SW 300 protein column (Waters Associates, Milford, MA) in a 100 mM KPO_4 buffer (pH 7.0).

Samples were prepared for the HPLC by first reconstituting the lyophilized,crude, stabilized venom to its original volume using sculpin saline. The samples were filtered free of particulates using disposable centrifugal filtration tubes (Nylon-66, Rainin, Woburn, MA) with 0.45 micron filters.

RESULTS

IN VITRO ISOLATED HEART EXPERIMENTS

1) Controls

Before any of the stabilization assays or pharmacological experiments were begun, three control hearts were mounted on the Langendorff perfusion apparatus for a three hour interval. These control hearts served to test the overall viability of the sculpin hearts, and to establish quantitatively the natural changes in the measured heart parameters over time due to fatigue, nutrient depletion, etc. These values were then compared to changes in the heart parameters, from drug or venom administration.

Initially, all hearts mounted to the perfusion apparatus exhibited a strong tachycardia, as compared to stabilized *in vitro* and *in vivo* heart rates, which stabilized to consistent values by 30 to 45 minutes. Heart rate consistently decreased over the test period dropping 52% by three hours. Most of this decrease (70%) occurred in the first 30 minutes. Pulse pressure increased initially, but decreased by about 10% every 30 minutes. Cardiac output generally followed the trend of the heart rate. The greatest proportion of change in the heart parameters occurred within the first 30 minutes, and the values were the most stable from 30 minutes to 2 hours. Thus, in all of the experiments utilizing the isolated heart preparation, any perturbation (drug or venom administration) was done between 30 and 120 minutes. These results concerning heart parameter stability are in agreement

with the literature (Stuart, 1981; Stuart _et al._, 1983).

2) Stabilization Study - Protease Inhibitor Cocktail

Fresh lionfish venom extracts, prepared in the 0.9% NaCl solution and administered in a low dose (40 μg protein/ml), produced a positive chronotropic effect (Fig. 8A). Heart rate increased an average of 50% (± 1.6%) in the first five minutes after venom administration, and slowly dropped by 35 minutes to 28% (± 1.4%) above control values. Cardiac output also increased with heart rate 31% (±0.9%), and pulse pressure was not affected. Venom from the same preparation (in the 0.9% NaCl solution), but stored at -20°C for 20 hours, failed to produce any change in the heart parameters (Fig. 8B) at the same and at higher concentrations (range 40 μg to 1300 μg protein/ml).

Fresh venom extracts prepared in the protease inhibitor cocktail (PIC) and administered in the same concentration (40 μg protein/ml), resulted in a considerably stronger effect (Fig. 8C). Heart rate increased an average of 43% (± 1.4%) from control values in the first 12 seconds, and decreased rapidly to complete cardiac standstill by 36 seconds after administration. Changes in cardiac output generally followed the trend of heart rate. Pulse pressure decreased to the baseline in 12 seconds. The PIC prepared venom extracts (Fig. 8C) possessed more activity and seemed to be protected from proteolysis as compared to the control venom extracts prepared only in the 0.9% NaCl solution (Fig. 8A).

Venom from the same PIC preparation, but stored at -20°C for 48 hours (Fig. 8D), produced effects similar to that of the

Figure 8. Representative chart recordings showing the effects of fresh, stored, and chemically stabilized lionfish venom on the isolated buffalo sculpin heart. Recordings show heart rate (time) and pulse pressure (cm water). Direction of recording is left to right, and vertical arrows indicate the point at which a one ml bolus of lionfish venom was administered.

A. Fresh venom extract prepared in the 0.9% NaCl solution and administered in a dose of 40 μg protein/ml

B. Venom extract from same preparation and concentration as in A, but tested after 20 hours of storage at -20°C

C. Fresh venom extract prepared in the protease inhibitor cocktail (PIC) and administered at the same dose (40 μg protein/ml)

D. Venom extract from same preparation and concentration as in C, but tested after 48 hours of cold storage

* The PIC administered alone had no effect.

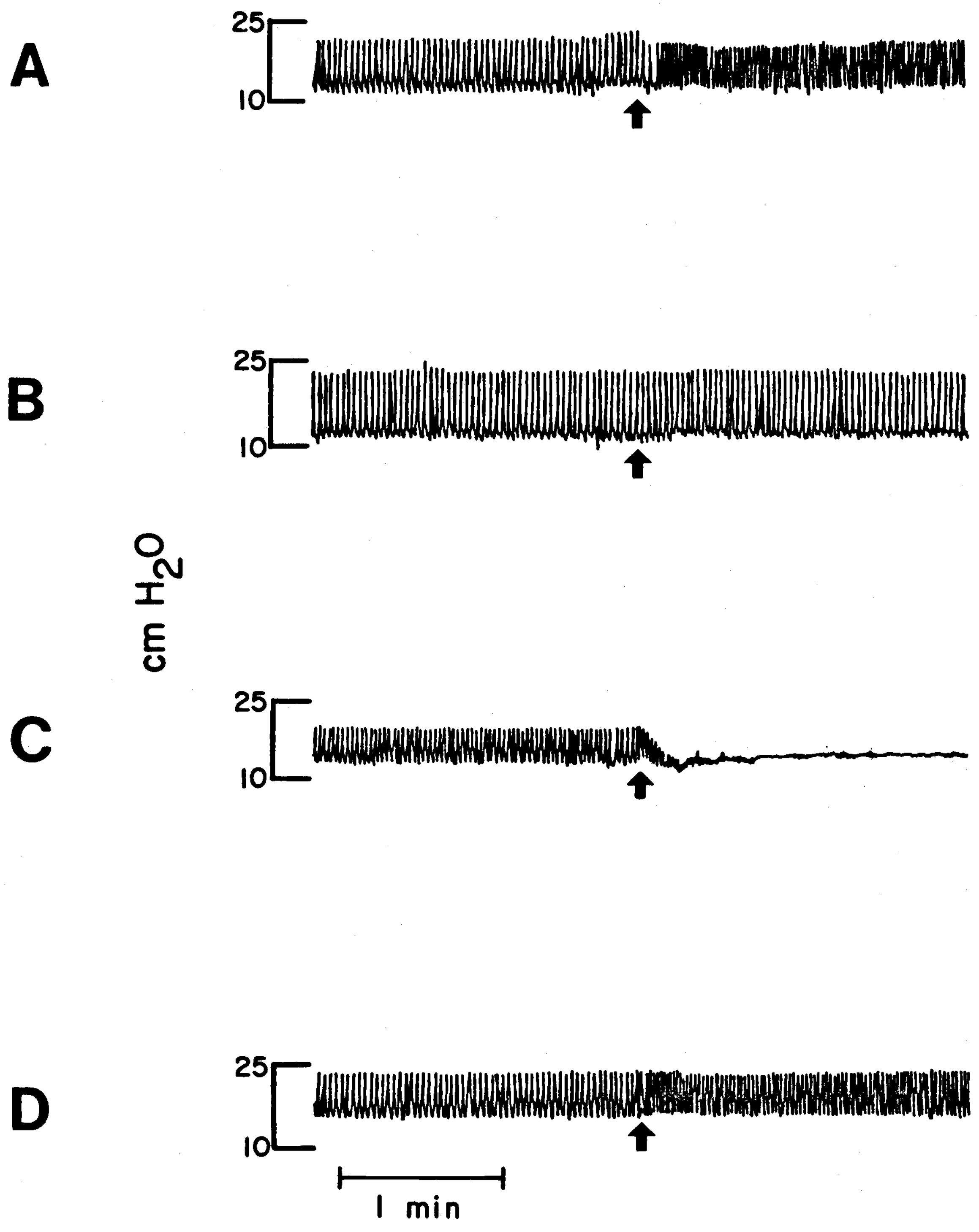


Figure 8

non-protected fresh venom. Heart rate increased an average of 47% (± 0.6%) from pre-administration control values. Cardiac output also increased with heart rate to 29% (± 1.6%) above control. No change in pulse pressure occurred. Administration of the PIC alone produced no significant changes in the heart parameters.

3) Stabilization Study - α2-Macroglobulin

Lionfish venom extracts prepared in the presence of the plasma protein/protease inhibitor, α_2-macroglobulin (1 μg/ml), were without effect on the isolated sculpin heart at any concentration when tested fresh, or after lyophilization and -85°C storage. α_2-macroglobulin had no effect on the isolated heart when administered alone. α_2-macroglobulin had no stabilizing effects on lionfish venom activity whatsoever, and possibly enhanced the temporal decline of this activity.

4) Long-term Stabilization Study - PIC

Fresh lionfish venom extracts, prepared in either the 0.9% NaCl solution or the protease inhibitor cocktail (PIC), when administered in higher doses (100-200 μg protein/ml) did not produce the expected tachycardia as it did with the lower (40 μg protein/ml) dose (Fig. 8). These higher doses produced a characteristic, and very reproducible, negative chronotropic effect (Fig. 9 A,C,&D). Heart rate decreased an average of 52% (± 1.1%) in the first ten minutes following a 1.0 ml bolus administration of the venom in this dose range. Cardiac output dropped an average of 31% (± 1.3%) coinciding with the decrease in heart rate, and pulse pressure was not affected.

Although the heart parameter values were averaged and compared at plus and minus ten minutes from the point of venom administration, this bradycardia, like the tachycardia produced by the lower doses (Fig. 8), was not a transient effect. The bradycardia produced by a medium dose of lionfish venom, and administered in only a one ml bolus, often lasted up to 45 minutes. Values either remained close to the post-administration ten-minute average, or decreased further before returning to values not significantly different from the pre-administration controls. In the case of the representative heart presented in Figure 9D, heart rate and cardiac output continued to decrease to an average of 62% and 56%, respectively, below pre-administration control values by 30 minutes. Pulse pressure, however, still did not change significantly.

In 75% of these experiments, administration of the venom would cause an immediate cardiac standstill which would last for 30 seconds to one minute (Fig. 9 C&D). Very high doses (1.0 mg protein/ml) produced complete and irreversible cardiac standstill (Fig. 10).

These same venom extracts, prepared in either the 0.9% NaCl solution or the PIC, were placed on ice (0-4°C) for two hours before being tested on the isolated buffalo sculpin heart. The venom extracts prepared in the 0.9% NaCl solution produced no significant changes in the heart parameters when administered after this two hour period (Fig. 9B). The PIC-prepared extracts, which were placed on ice for the same two hour interval showed no loss of activity (Fig. 9C).

Figure 9. Representative chart recordings showing effects of higher doses and of long-term storage of the lionfish venom on the isolated buffalo sculpin heart. Recordings show heart rate (time) and pulse pressure (cm water). Direction of recording is left to right, and vertical arrows indicate the point at which a one ml bolus of lionfish venom was administered.

A. Characteristic bradycardia produced by fresh, lionfish venom (100 μg protein/ml) which was prepared with the protease inhibitor cocktail (PIC)

B. Administration of a 200 μg protein/ml dose of lionfish venom, prepared in the 0.9% NaCl solution only, which were stored at 0-4°C for two hours

C. Administration of a 200 μg protein/ml dose of lionfish venom, prepared in the PIC only, which were stored at 0-4°C for two hours

D. Administration of a 200 μg protein/ml dose of the same batch of lionfish venom as in C, but lyophilized, and stored at -85°C for 110 days before being reconstituted and administered

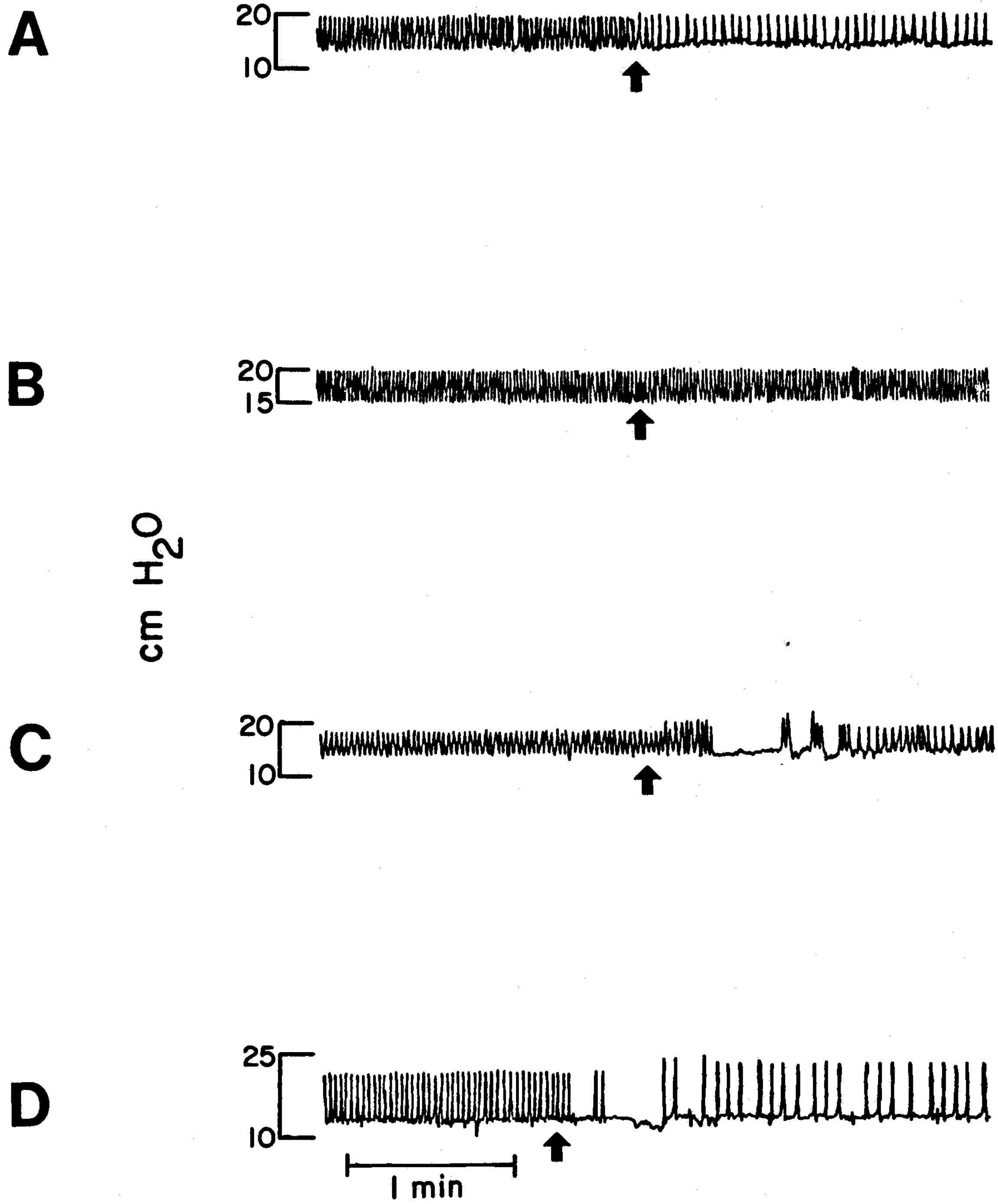


Figure 9

Aliquots of the venom extracts from this same preparation were lyophilized and stored at -85°C. These extracts were later tested on the isolated sculpin heart after 110 days of cold storage. The reconstituted venom extracts from the 0.9% NaCl solution preparation had no effect on the isolated hearts. The reconstituted PIC-prepared venom extracts exhibited essentially the same amount of activity as before storage (Fig. 9D). Thus, lionfish venom, prepared in the presence of the protease inhibitor cocktail, lyophilized, and stored at -85°C, seems to remained stable for a relatively long period of time.

5) Overall Effects of Lionfish Venom on the Isolated Heart

As presented in the above PIC-stabilization study, low doses (40 μg protein/ml) of lionfish venom, either fresh extracts or those chemically stabilized with the protease inhibitor cocktail and reconstituted from the lyophilized state, produced a positive chronotropic effect with a concomitant increase in cardiac output, and no effect on pulse pressure (Figs. 8A, 8D, 10B; Table 1).

Higher doses (100-200 μg protein/ml), although still in the sublethal range, produced considerably different and completely opposite effects on the isolated heart. Fresh venom extracts, or those stabilized with the PIC and reconstituted from lyophilization and cold storage, when administered in these higher doses, produced a distinct negative chronotropic effect. Cardiac output generally decreased with the drop in heart rate, and pulse pressure was not significantly affected (Figs. 9A, 9C, 9D & 10C; Table 1).

Figure 10. Representative chart recordings summarizing the overall effects of the lionfish venom on the isolated buffalo sculpin heart. Recordings show heart rate (time) and pulse pressure (cm water). Direction of recording is left to right, and vertical arrows indicate the point at which a one ml bolus of venom was administered.

A. Heart rate and pulse pressure recording of a normal, rhythmic sculpin heart before drug or venom administration

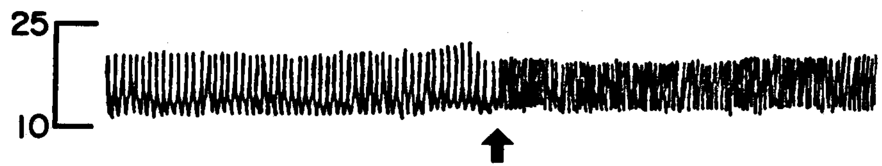
B. Characteristic tachycardia produced by low doses (40 μg protein/ml) of lionfish venom

C. Characteristic bradycardia produced by mid-range doses (100-200 μg protein/ml) of lionfish venom

D. Complete and irreversible cardiac standstill produced by high doses (750-1300 μg protein/ml) of lionfish venom

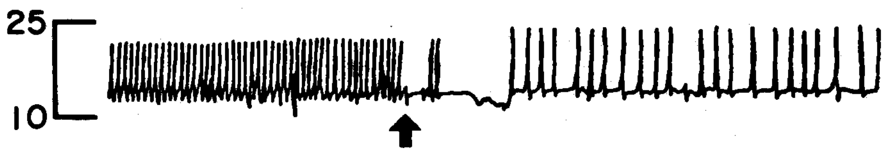
A

B



cm H_2O

C



D

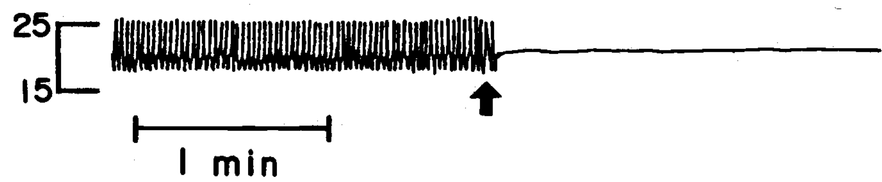


Figure 10

TABLE 1

SUMMARY OF THE EFFECTS OF *PTEROIS VOLITANS* VENOM ON THE ISOLATED BUFFALO SCULPIN HEART

Dose (μg protein/ml)	Heart Rate	Pulse Pressure	Cardiac Output
(low) 40	↑49%	N.S.	↑30%
(med) 100-200	↓52%	N.S.	↓31%
(high) 750-1300	↓100%	↓100%	↓100%

- the % change values listed are significantly different from the control hearts at $p<0.01$

- N.S. = no significant change in the parameter as compared to the controls at $p>0.05$

Much higher doses (range 750-1300 μg protein/ml) caused complete and irreversible cardiac standstill within 30 seconds of venom administration (Fig. 10D; Table 1).

IN VIVO CARDIOVASCULAR EXPERIMENTS

The effects of lionfish venom on the cardiovascular system of live buffalo sculpin are depicted in figure 11C. Sub-lethal doses (range 75-200 μg protein/kg) produced essentially the same strong, non-transient, negative chronotropic effect as the mid-range doses (100-200 μg protein/ml) administered to the isolated sculpin heart. *In vivo* heart rate characteristically decreased an average of 53% below the pre-administration control values, and lasted 20 minutes or longer. Additionally, administration of lionfish venom, *in vivo*, produced a positive ionotropic effect heretofore not seen associated with the venom. Blood pressure usually increased beyond instrument calibration, which had been calibrated to the normal maximum expected pressure of 50 cm H_2O (Fig. 11C). In one of the later experiments utilizing this preparation, pressure monitoring equipment was re-calibrated to a maximum pressure of 80 cm H_2O, and lionfish venom administration was found to increase blood pressure an average of 78% (68 cm H_2O). The temporal aspect of this positive ionotropy was more variable than the other measured cardiovascular parameters and could last from seven minutes up to 64 minutes.

Figure 11. Representative chart recordings showing the effects of stabilized lionfish venom on the cardiovascular system of live, cannulated buffalo sculpin in the *in vivo* preparation. Recordings show heart rate (time) and blood pressure (cm water). Direction of recording is left to right, and vertical arrows indicate the point at which a stimulus or the venom was administered.

A. Characteristic recording of heart rate and blood pressure from a successfully cannulated, non-perturbed buffalo sculpin

B. Typical, transient response bradycardia and pressure decrease elicited by a visual stimulus. This response, along with the administration of low doses of the control drugs acetylcholine and norepinephrine, is used to test the usability of the *in vivo* preparation

C. Sub-lethal doses (range 75-200 μg protein/kg) of lionfish venom characteristically produced an extreme and long lasting bradycardia, and a large increase in blood pressure. (Dose for this recording = 100 μg protein/kg)

D. High doses (500-750 μg protein/kg) of lionfish venom caused complete and irrecoverable cardiac standstill and death. (Dose for this recording = 750 μg protein/kg)

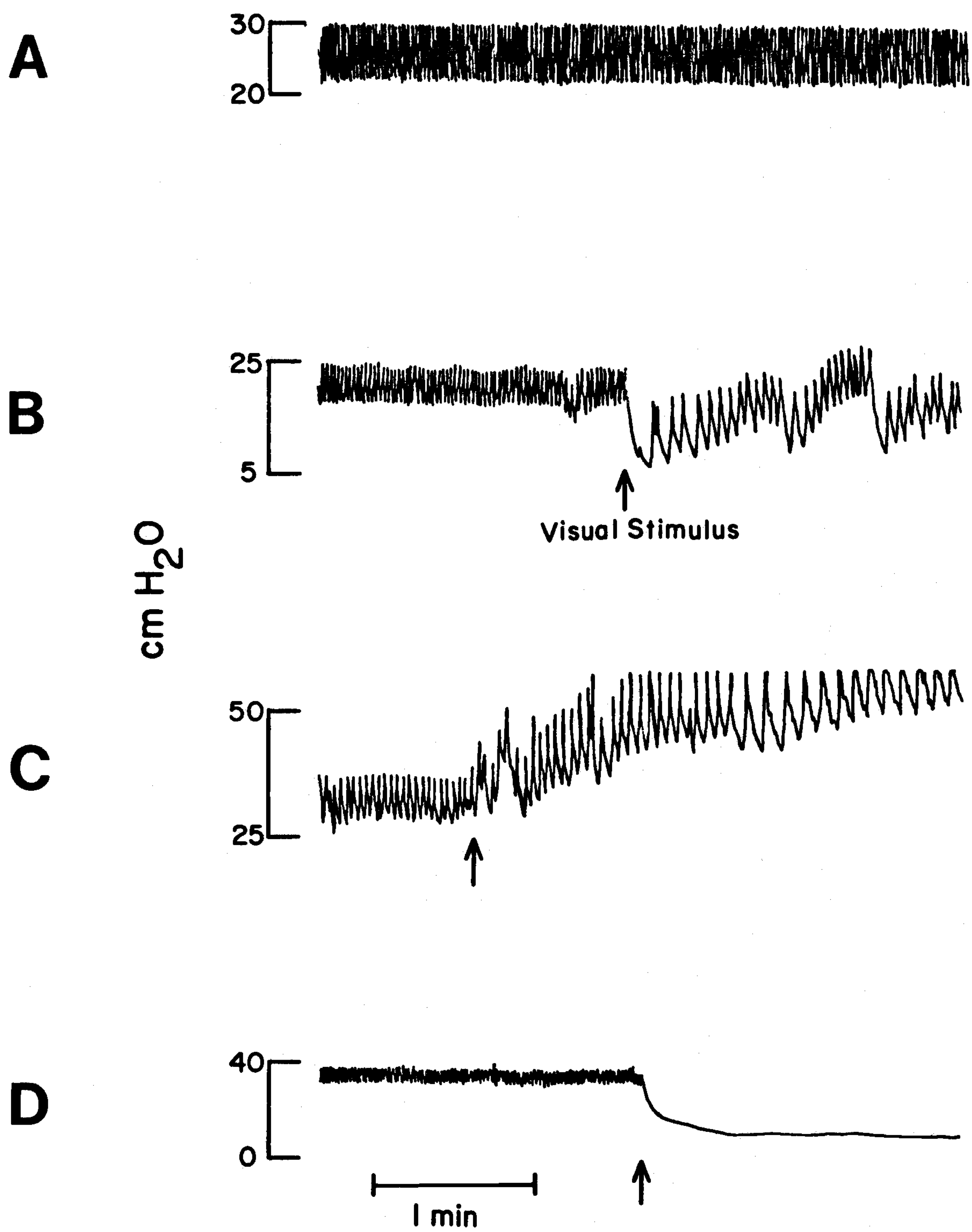


Figure 11

Higher doses of the lionfish venom (500-750 μg protein/kg) administered to live buffalo sculpin in this preparation produced complete cardiac standstill and death (Fig. 11D).

IN VITRO ISOLATED TAIL/INTACT VASCULATURE EXPERIMENTS

Figure 12 depicts the results of the administration of norepinephrine, acetlycholine, and the lionfish venom to the isolated fish tail preparation. The results indicate that the venom produces a substantial increase in peripheral resistance, which is assumed to be due to a vasoconstrictor property of the venom. Relative pressure increased beyond instrumentation range when lionfish venom was administered, and did not return to measurable range after a 30 minute interval, or after subsequent administration of acetylcholine.

BIOASSAY

Table 2 shows the LD_{50} determination of the reconstituted, lyophilized, PIC-treated crude venom extracts on the buffalo sculpin. Control specimens, which were injected with either sculpin saline or the PIC alone, displayed no adverse effects. The resultant LD_{50} value of 200 μg protein/kg determined by the multiple sample up-and-down method of bioassay is significant when compared to the sham treatments at $p<0.05$.

Symptoms exhibited by the sculpins after a direct cardiac injection of lethal doses of lionfish venom included increased motor activity and increased respiratory rate within one minute,

Figure 12. Representative chart recordings showing the effects of stabilized lionfish venom, and the effects of the control pharmacological agents, on the isolated tail/ intact vasculature preparation. Recordings show only relative changes in pressure over time as indicated by + or -. Direction of recording is left to right, and vertical arrows indicate the point of drug or venom administration.

A. Transient increase in relative pressure caused by a low dose (10 μg/kg) of norepinephrine

B. Transient decrease in relative pressure caused by a low dose (2 μg/kg) of acetylcholine

C. Non-transient and irreversible increase in relative pressure produced by the administration of stabilized lionfish venom (100 μg protein/kg)

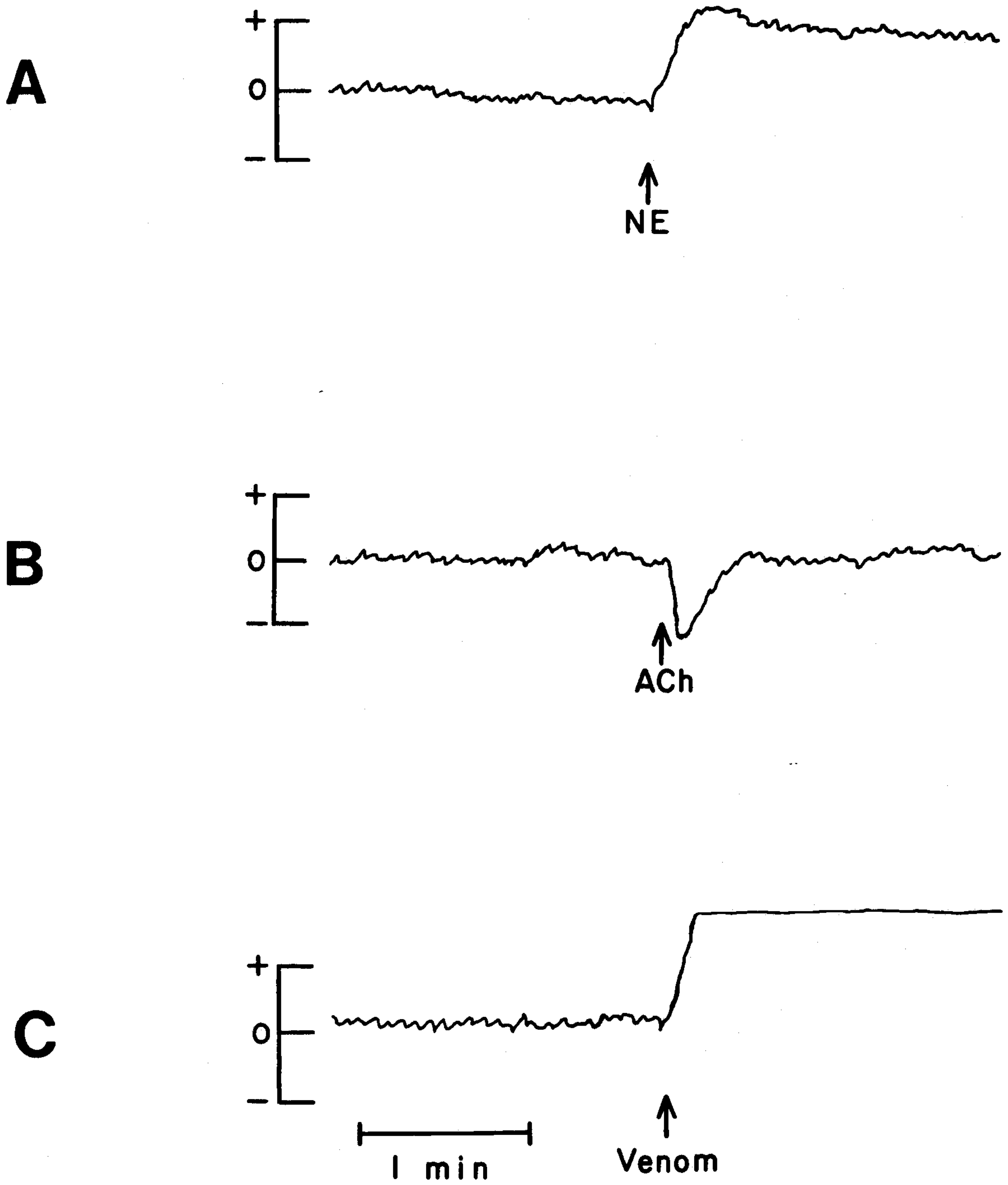


Figure 12

TABLE 2

LD_{50} DETERMINATIONS OF LYOPHILIZED, P.I.C.-TREATED *PTEROIS VOLITANS* VENOM IN THE BUFFALO SCULPIN

Dose (µg protein/kg)	#Deaths/ #animals tested	% Death
750	2/2	100%
500	2/2	100%
375	2/2	100%
250	3/4	75%
200	2/4	50%
150	1/4*	25%
100	0/2	0%
75	0/2	0%

Lionfish Venom LD_{50} in Buffalo Sculpin:

200 µg protein/kg body weight

* = surviving specimens exhibited symptoms associated with lethal doses (see text).

followed by rapid and convulsive twitching of all of the fins independent of each other. Death usually occurred within 20 minutes.

LIVER FUNCTION

The results of the liver function experiment using cannulated buffalo sculpin are presented graphically in Figure 13. The doses of lionfish venom, in this experiment, were inadvertently not normalized to the fish body weight. Consequently, sculpin #1 received a dose of 225 μg protein/kg, while sculpin #2 received 180 μg protein/kg (20% less than sculpin #1). In the two animals that survived surgery, i.v. administration of lionfish venom caused a substantial decrease in the sulfobromopthalein (BSP) elimination rate. Sculpin #1 showed a seven-fold decrease, while sculpin #2 showed only a two-fold decrease. In the case of sculpin #1, the elimination rate was reduced to almost zero. This large difference in elimination rates between the two fish is most likely dose related.

SDS GEL ELECTROPHORESIS

A separation of reconstituted lionfish venom on SDS gel electrophoresis is shown in Figure 14. From this experiment, it was determined that the lionfish venom protein(s) have subunit molecular weights primarily in the range of 116,000; 97,000; 66,000; 45,000 and 29,000.

Figure 13. Graphical representation of the BSP elimination rates from plasma in two specimens of buffalo sculpin. Graphs are natural log transformations of BSP concentration in sampled sculpin plasma vs time in minutes.

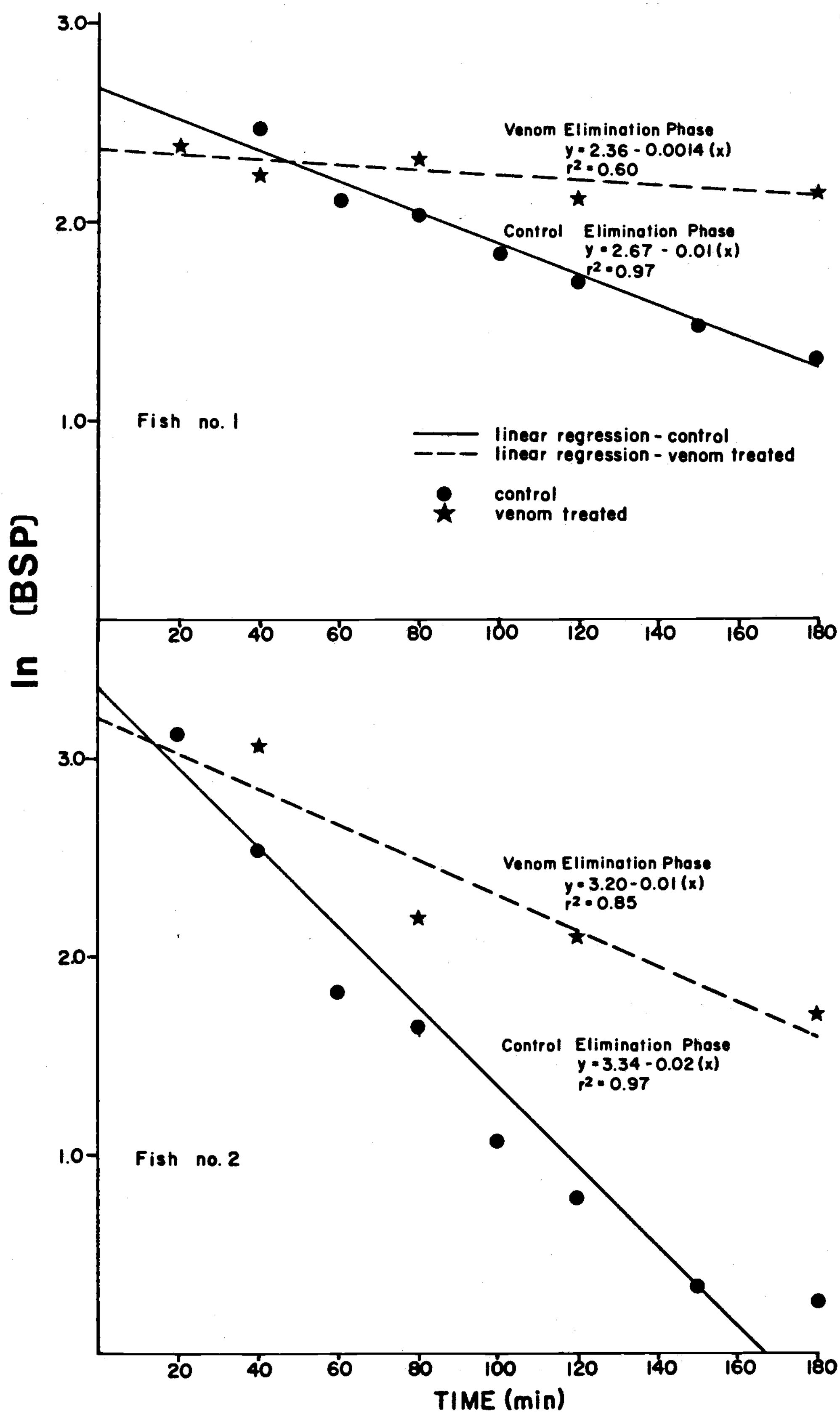


Figure 13

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The elution profile of a 20 µl sample of lyophilized, PIC-treated lionfish venom chromatographed on a Synchropak reverse phase column is shown in Figure 15. The initial peaks at 219 nm (fractions 1-10) were most likely produced by components of the protease inhibitor cocktail present with the venom, and were matched with peaks produced when the PIC was chromatographed alone. One ml fractions were collected and lyophilized, and subsequently reconstituted with sculpin saline and injected into juvenile buffalo sculpin. None of these fractions produced any observable adverse effects in these fish.

Figure 16 shows the elution profile of a 25 µl sample of fresh crude lionfish venom chromatographed on the Waters SW-300 protein column. This HPLC experiment was analytical only, and fractions from this elution were not collected.

Figure 14. Photograph of a representative gel electrophoretic separation of stabilized lionfish venom in relation to molecular weight markers and the protease inhibitor cocktail (PIC) alone.

Key: A - Wells containing Sigma molecular weight markers #SDS-7.

B - Wells containing Sigma molecular weight markers #SDS-6H.

C - Wells containing stabilized lionfish venom reconstituted from lyophilization and cold storage.

D - Wells containing the PIC alone.

* - molecular weight values of marker subunits are shown at the sides of the gel.

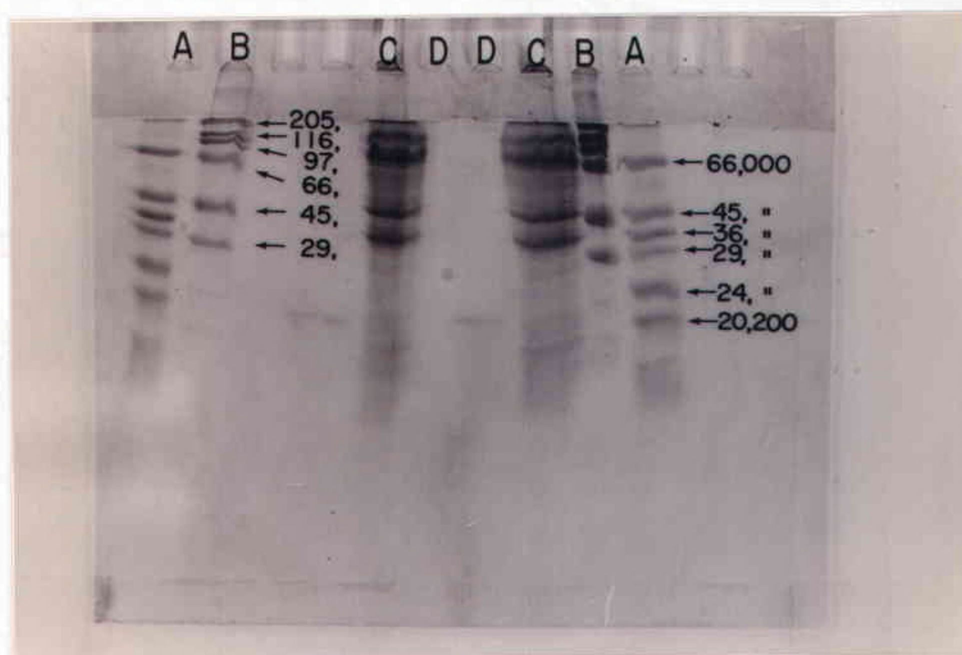


Figure 14

Figure 15. Elution profile of 20 μl of lyophilized, PIC-treated Pterois volitans venom containing 20 μg protein, chromatographed with HPLC using a Synchropak reverse phase column.

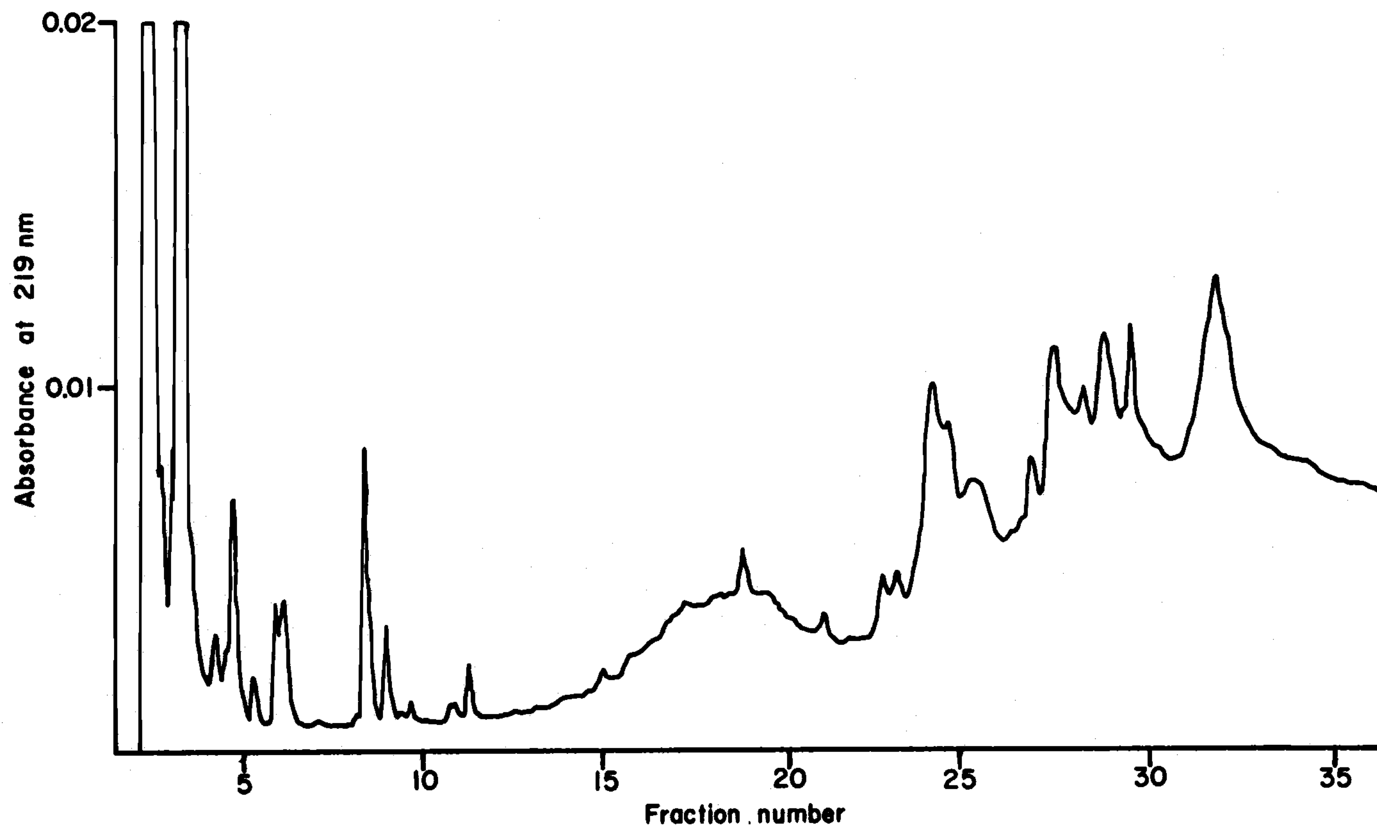
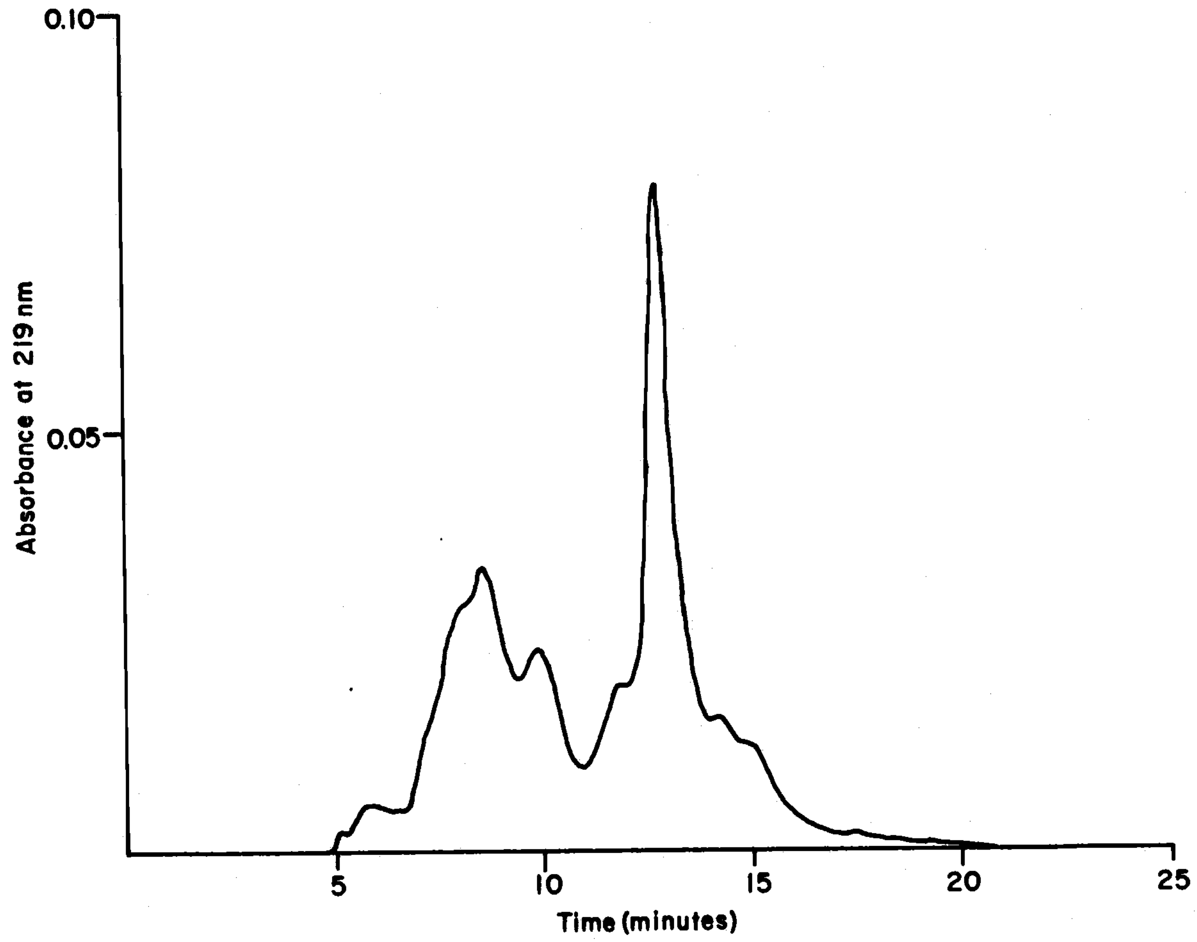


Figure 15

Figure 16. Analytical HPLC map of fresh crude lionfish venom containing 25 μg protein, chromatographed using a Waters SW-300 protein column.

Figure 16



DISCUSSION

VENOM EXTRACTION PROCEDURES

The various methods of venom extraction attempted without a successful yield (see Methods and Appendix) indicates that the chosen method, that of complete venom apparatus (spines and associated tissue) removal, homogenization, and centrifugation, is preferred. Although this method does include some extraneous material in the homogenates (bone fragments, skin tissue, etc.), far less contamination and a much larger venom yield is achieved, than with the other methods attempted. This preferred method was a modification of the venom extraction procedure developed by Saunders and Taylor (1959).

IN VITRO ISOLATED HEART CONTROLS

The initial tachycardia at the beginning of all of the isolated sculpin heart perfusions was most likely due to the liberation of catecholamines during the surgical excision of the hearts. This hypothesis, proposed by Stuart _et al_. (1983), is supported by washout studies. This catecholamine induced tachycardia diminished during the first 30 minutes of perfusion, and heart rate stabilized after this interval.

STABILIZATION STUDIES

The addition of the protease inhibitor cocktail (PIC) to the excised venom apparatus prior to homogenization was successful in stabilizing the activity of the lionfish venom, as evidenced by

the isolated sculpin heart assays. Venom preparation in the presence of the PIC seems to successfully minimize the proteolytic degradation of the venom, and the toxic activity is retained.

This initial stabilization study tested the effects of the PIC-prepared lionfish venom after only 20 hours of storage at -20°C. In the subsequent PIC stabilization experiments, lyophilization and -85°C storage were added to the venom preparation protocol, and successful stabilization of the venom was achieved after a storage period of 110 days (the longest interval tested).

The use of such protease inhibitor cocktails to minimize proteolytic degradation is not unique. For example, the use of a PIC is standard where protection from proteolytic activity is necessary, such as in receptor binding experiments (Chang et al., 1977; Hartshorne and Catterall, 1984). Russell (1965) has noted a remarkable similarity in the pharmacological properties of a variety of venoms from marine fishes and believes that the venoms are similar chemically. The use of protease inhibitors, as introduced here and in Choromanski et al. (1984), successfully stabilizes lionfish venom and possibly may overcome the instability problems associated with other fish venoms.

It was along this same line of thought, regarding proteolytic degradation as the cause of fish venom instability, that it was decided to test α_2-macroglobulin as a possible venom stabilizing agent. α_2-macroglobulin has been claimed to be a universal protease inhibitor, capable of inactivating all known endoproteases (Starkey and Barrett, 1977). α_2-macroglobulin does not inhibit

proteolytic enzymes in the usual sense, but rather it "traps" them via a unique reaction mechanism. This "trap hypothesis" is described in detail by Barrett and Starkey (1973). While this hypothesis apparently explains the mechanism by which α_2-macroglobulin inactivates proteases, recent evidence (Travis and Salvesen, 1983) suggests that at least two additional types of bind reactions may be operating, including: 1) a covalent linking of proteases and other molecules containing nucleophilic groups, and 2) a noncovalent, nonsteric adherence reaction with a number of other proteins and other molecules, unrelated to proteolytic activity.

Thus, the venom protein(s) itself may becoming inactivated via this nonsteric adherence reaction, which would help to explain both the lack of any stabilizing effects offered by α_2-macroglobulin, and the rapid loss of all activity in the α_2-macroglobulin venom preparations.

Carlson (1972) was able to achieve a partial stabilization of Scorpaena guttata venom for up to ten days by adding Cleland's reagent (1 mM), an effective sulfhydryl reagent, to the venom after lyophilization. He also found that diisopropylfluorophosphate (1 mM), also a protease inhibitor, had a slight stabilizing effect on this scorpionfish venom.

Saunders and Taylor (1959) found that lyophilization and cold storage (-20°C) helped extracted lionfish (Pterois volitans) venom to retain 40% to 90% of its activity for over one year. They admit, however, that a precise determination of stability was not possible in the field.

OVERALL PHARMACOLOGICAL EFFECTS OF LIONFISH VENOM

Discussion of the overall pharmacological effects of lionfish venom, on the various cardiovascular survey preparations presented here, is difficult for a variety of reasons. First, there is only one other published report of controlled pharmacological experiments with lionfish venom for comparison (Saunders and Taylor, 1959), and this work was quite preliminary. Russell (1965) and others, however, have stated that there is a remarkable similarity in the pharmacological properties of a variety of fish venoms, particularly the scorpaenids, and that comparisons can be made between them. Thus, in addition to comparing the present results to the single work of Saunders and Taylor (1959), comparisons will also be made to the pharmacological effects produced by other fish venoms.

The present work is also the first controlled attempt at using a species of fish (the natural intended target) as the experimental animal for the testing of the venom effects. Comparison of these results to those of other works using the same or other fish venoms on mammalian experimental animals, may or may not be valid. For example, Russell and Long (1961), and Russell and Emery (1960) have studied the effects of a variety of fish venoms (not including the lionfish) on neuromuscular transmission, and found none of the fish venoms to have any effect on the mammalian species tested. However, the symptomology produced from lionfish venom administration into the live buffalo sculpin (that of the rapid and convulsive twitching of the fins independent of each other) indicates that there may indeed be some neuromuscular

effects. Whether or not these exhibited effects are due to a property unique to lionfish venom, or due to the fact that we are using a species of fish (the intended target) as the experimental animal, is not known.

Austin *et al*. (1961) have published data indicating that stonefish (*Synanceja*) venom does produce a neuromuscular blockade in rats *in vivo*. Future work with lionfish venom should include tests for effects on neuromuscular transmission in both aquatic and mammalian species.

In the isolated buffalo sculpin heart low doses of lionfish venom consistently elicited a positive chronotropic response. Interestingly, larger doses produced a distinct negative chronotropy. Very high doses caused complete and irreversible cardiac standstill and pulse pressure did not change at any dose level. These rate changes in the isolated heart were surprisingly non-transient from a low volume bolus administration. These results are in contrast to those obtained by Saunders and Taylor (1959) using anesthetized rabbits for lionfish venom administration. They found no change in cardiac rate at any dose administered; however, this may have been due to the depression of cardiac reflexes produced by anesthesia.

The present results actually are somewhat similar to those obtained by Russell and Van Harrelveld (1954) and Russell *et al*. (1958) using the venom of the round stingray, *Urolophus halleri*. They found this venom to exert a very deleterious effect on the mammalian cardiovascular system in general, and specifically they found a direct effect on the cat heart muscle. Their stingray

venom produced both increases and decreases in heart rate, and high doses causes complete and irreversible cardiac standstill. They concluded that the venom directly affected the myocardium and the normal pacemaker.

Studies on another venomous fish, the weever (Trachinus) also revealed results similar to the present results with lionfish. Pohl (1893) and Russell and Emery (1961) found marked decreases in heart rate after administering weeverfish venom to cats.

Carlson (1972) found profound heart rate changes in cats and dogs from the administration of scorpionfish (Scorpaena guttata) venom, and high doses produced complete cardiac standstill.

In the isolated tail/intact vasculature preparation, the administration of lionfish venom produced a distinct and non-transient increase in peripheral resistance. It can be assumed that this was due to a vasoconstrictor property of the venom, although the possibility of indirect release of autopharmacological substances and adrenergic mechanisms cannot be ruled out.

These results also are in contrast to those obtained by Saunders and Taylor (1959) also investigating the effects of Pterois volitans venom. They found the major effect produced by the venom in anesthetized rabbits to be a marked hypotension they suggested was caused by a peripheral vasodilation.

These results are, however, in agreement with the literature utilizing other fish venoms. Russell et al. (1958) found that high concentrations of stingray (Urolophus) venom caused a marked vasoconstriction of the large arteries and veins, as well as the

arterioles in the mammalian species tested. Russell and Emery (1961), too, found that weeverfish venom produced a transient vasoconstriction or vasodilation depending upon the amount injected into cats. Austin *et al*. (1961) found stonefish (*Synanceja horrida*) venom to cause vasoconstriction in the isolated perfused rabbit hind limb, and Saunders (1960) and Saunders *et al*. (1962) found the venom from this species to produce changes in peripheral resistance and blood flow. Carlson (1972) found that the most consistent finding associated with scorpionfish (*Scorpaena*) venom is a pulmonary artery hypertension, preceding the overall systemic arterial hypotension, which he concluded was due to a constriction of the pulmonary arterioles.

Lionfish venom produced both a negative chronotropic effect and a positive ionotropic effect in the *in vivo* preparation using cannulated buffalo sculpin. This bradycardia and hypertensive effects do not agree with what Saunders and Taylor (1959) found with venom from this same species when tested on anesthetized rabbits. They could not detect any cardiac rate changes produced by the administration of lionfish venom and they concluded that the major effect produced by this venom is a hypotension. Other researchers, as stated previously, have found similar results with other fish venoms, that are in general agreement with those presented here. Thus, the potential for both changes in heart rate, and a vasoconstrictor property associated with fish venoms exists, and these changes have been documented in accounts utilizing other related species of venomous fish.

Why the present results obtained with lionfish venom differ so much from the only other published account of the pharmacological effects of this same venom (Saunders and Taylor, 1959) is not known. It is possible that the present utilization of a species of fish is a more sensitive measure of the lionfish venom toxicities. This difference could also be attributable to the fact that Saunders and Taylor's venom preparation was done under less than optimal, non-laboratory conditions. Whatever the reason, it does serve to demonstrate the need for more research with the venom of the lionfish.

BIOASSAY

The results of the bioassay of lionfish venom on the buffalo sculpin indicate an intravenous LD_{50} of 200 μg protein/kg body weight. This value is considerably lower than the 1.1 mg protein/kg body weight i.v. LD_{50} of lionfish venom obtained by Saunders and Taylor (1959) using mice. This difference may be partially due to the "in-field" methods of venom extraction and preparation used by these authors, or due to a species-sensitivity difference. Indeed, many workers have obtained considerably different LD_{50} values using the same fish venom preparation on different species of test organisms. For example, Carlson (1972, *et al.*,1973) found a wide range of lethality levels in several different animals with an intravenous administration of scorpionfish (*Scorpaena guttata*) venom ranging from 300 μg protein/kg in rabbits, to 2.6 mg protein/kg in mice. The cats and dogs tested had LD_{50}'s within this range,

and shore crabs and the octopus were resistant to massive doses of the scorpionfish venom. It is also possible that fishes in general (the intended target) are more sensitive to the adverse cardiovascular and lethal effects of fish venoms, than are mammalian species. Further experimentation would characterize this possible species sensitivity difference with lionfish, and other piscine venoms.

LIVER FUNCTION

To interpret the results of the liver function experiment, the kinetics of a two-compartment model for bolus injection was used. This two-compartment model suggests that sulfobromopthalein (BSP) elimination is determined by both the rate of BSP distribution to and from the peripheral compartment, and the active transport of BSP to the bile. Since it has been suggested that lionfish venom does cause a vasoconstriction of the peripheral vasculature, as evidenced by the *in vivo* and isolated tail preparation results, it can be hypothesized that the lionfish venom is possibly causing a reduction in hepatic perfusion by producing a constriction in the vasculature leading to the liver. This hypothesis is supported by the fact that BSP elimination is perfusion limited in mammals, and more than 95% of the BSP which reaches the liver in one pass is excreted into the bile.

Lionfish venom does indeed affect normal BSP elimination from plasma, however, the mechanism of action (most likely due to the cardiovascular effects) has not been confirmed. This experiment

was not designed to differentiate between the potential (direct) liver effects and the known (indirect) cardiovascular effects. If liver toxicity induced by lionfish venom administration is to be investigated further, experimentation involving the monitoring of BSP elimination in the bile should be included.

SDS GEL ELECTROPHORESIS

Stabilized lionfish venom was separated by SDS gel electrophoresis. Analysis of the electrophoretic results reveals that lionfish venom has components, or subunits, that correspond to molecular weight markers in the range of 29,000 to 116,000.

Saunders and Taylor (1959) did not attempt any type of chemical separation with their lionfish venom, however, the present results indicating that lionfish venom is generally composed of high molecular weight components is in agreement with the literature (Russell and Brodie, 1974; Halstead, 1978).

Very few electrophoretic separations have been performed with fish venoms for comparison, and each technique is somewhat different. Saunders and Tokes (1961) utilized starch-gel electrophoresis in a pH 8 phosphate buffer for the analysis of stonefish (*Synanceja horrida*) venom. They found 7-10 different components of the venom, and only one of these components had any lethal activity when injected into mice. This separation increased the venom potency two-fold from the original sample.

Taylor (1963) and Carlson (1972) both used cellulose acetate strip electrophoresis to analyze scorpionfish (*Scorpaena guttata*)

venom, and found lethal activity to be associated with moderately negatively-charged components of high molecular weight at pH 7.4-8.5.

In the present work, a substantial amount of venom material was retained in the stacking gel, most likely due to a physical clogging of the gel from the large sample. Future electrophoretic separations of lionfish venom may need to utilize tube or slab gel electrophoresis both analytically and preparatively. These techniques utilize thicker gels with greater surface area which would solve any clogging problems, and which would yield larger concentrations of separated venom components for the determination of lethal activity. Gel electrophoresis offers the potential for rapid separation,concentration, and purification of fish venoms.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Fractionation of stabilized lionfish venom by High Performance Liquid Chromatography, utilizing a Synchropak reverse-phase column, revealed a number of large peaks, measured at an absorbance of 219 nm, toward the end of the elution profile (fractions 18-33). These peaks represent components of lionfish venom which are much more hydrophobic than the low molecular weight constituents of the protease inhibitor cocktail (PIC) which eluted first. By utilizing a reverse-phase column, a greater resolution of venom components was achieved. The reverse-phase separation yielded 18 distinct peaks, even after subtracting those produced by the PIC, whereas the Waters SW-300 protein column yielded only three peaks.

The reason that the reconstituted venom fractions (eluted from the 20 μl sample of crude venom on the reverse-phase column) did not produce any deleterious effects when injected into buffalo sculpin is not known. Russell and Brodie (1974) have noted that some components of fish venoms, when separated from the crude mixture, have no known pharmacological effect. They suggest that there may be some synergistic actions of whole venoms that are not present upon separation into the various fractions. Russell also postulates that the biological properties of some of these fractions may have played a role in the evolutionary past, but are no longer an essential part of the venom in the animal's present niche. Alternatively, it is also possible that the amount of the recovered samples eluted from the column was not sufficient enough to produce any effect. Simply by injecting a larger amount of sample onto the column at the beginning of the gradient, may be all that is needed to yield a sufficient amount of separated venom possessing lethal or toxic activity.

Lionfish venom was also separated on a Waters SW-300 protein column resulting in three protein peaks. The largest peak measured at 219 nm eluted after 13 minutes, and probably is a high molecular weight component of the venom. It is not known quantitatively how large these separated protein peaks are, without comparing them to similar separations of compounds of a known size.

This is the first report of a separation of any fish venom utilizing high performance liquid chromatography. This technique has the potential for detailed characterization and rapid purification

of known (histamine, serotonin, etc.) and unknown (toxic or lethal activity) fish venom components. Future work should be directed toward developing the application of HPLC techniques in venom research, specifically in the areas of venom purification and concentration, and quantitative analysis of venom components.

From the results of these various survey preparations, the effects of *Pterois volitans* venom on the cardiovascular system cannot be explained by a single mechanism. The varied results obtained from the *in vitro* and *in vivo* pharmacological preparations, and the nature of the symptomology associated with lionfish venom administration into the live buffalo sculpin, supports the concept that the deleterious effects of the crude, stabilized lionfish venom represent a combination of direct or indirect actions of the venom components. These actions may possibly involve effects induced by the release of autopharmacological substances, or perhaps responses mediated through neurogenic reflex mechanisms.

Some of the responses produced in the buffalo sculpin from the administration of lionfish venom are also somewhat similar to changes induced, in mammalian systems, by serotonin, histamine, catecholamines, and the kinins.

Serotonin (5-hydroxytryptamine) has been implicated as the pain producing substance in a number of fish venoms (Russell and Brodie, 1974). Carlisle (1962) found serotonin to be the "major if not the only pain producing substance" in the venom of the

lesser weeverfish, *Trachinus vipera*. Russell (1965), too, has found serotonin associated with the venom of the round stingray, *Urolophus halleri*, and it is a common component of many marine and terrestrial animal venoms. Serotonin has been found to be a very powerful vasoconstrictor of most vasculature, and has varying positive chronotropic effects on the heart (Page, 1958; Green and Kepchar, 1959; Douglas, 1980).

Histamine is known to cause pulmonary artery hypotension and the dilation of systemic arteries, however, it is also a *constrictor* of veins (Green and Kepchar, 1959; Kahlson and Rosegren, 1968). Histamine almost exclusively produces vasoconstriction of large vessels, tending to increase blood pressure (Douglas, 1980). Histamine has also been detected in increased levels in the blood after the administration of some snake venoms (Kahlson and Rosengren, 1968; Russell, 1967a, 1967b).

Catecholamines, and specifically epinephrine, are known to be potent vasoconstrictors in man, and they elevate systolic and mean pulmonary artery pressure (Witham and Flemming, 1951). Carlson (1972) found that *Scorpaena guttata* venom produced the release of catecholamines *in vitro*. Saunders (1960) and Saunders *et al*. (1962) found that stonefish (*Synanceja horrida*) venom produced changes in peripheral resistance and blood flow in the rabbit, and Austin *et al*. (1961) found the venom from this same species to cause vasoconstriction in the isolated perfused rabbit hind limb preparation. Weeverfish (*Trachinus draco*) venom was separated with paper chromatography, and one spot was identified as histamine

and the other as catecholamines which were later determined to be epinephrine and norepinephrine (Haaveldsen and Fonnum, 1963). Thus, although it is not known at present whether or not fish vasculature is innervated, and if so by what division of the autonomic nervous system, the possibility of venom induced vasoconstriction being due to adrenergic mechanisms cannot be ruled out.

The cardiovascular effects of the kinins are well documented and generally they cause a distinct vasodilation in most fine-resistance vessels. In contrast, the kinins have been found to cause constriction of large arteries and most veins (Douglas, 1980). In the isolated perfused rabbit lung preparation, however, bradykinin produces a marked vasoconstriction, as well as an effect on capillary permeability (Hauge *et al*., 1966). Many terrestrial animal venoms are known to release kinins *in vivo* and *in vitro* (Russell, 1967a,b, 1969; Kiniz, 1968), however, Austin *et al*. (1965) were unable to find any bradykinin-like activity associated with stonefish (*Synanceja*) venom when assayed on the isolated guinea pig ileum. No other fish venom has been specifically tested for this kinin or kinin-releasing actions (Carlson, 1972).

To summarize, it is hypothesized that although fish venoms, including that of the lionfish, may have *direct* cardiovascular effects, the possibility of autopharmacological responses must also be considered to help explain some of these cardiovascular changes. This hypothesis has been proposed for terrestrial animal venoms, as well as for other piscine venoms such as the scorpionfish (Carlson, 1972), and the round stingray (Russell *et al*., 1957,

1958; Russell and Van Harreveld, 1954). Since Russell (1965) has noted such a remarkable similarity in the pharmacological properties of the venoms of the stingray (Urolophus), the weever (Trachinus), the scorpionfish (Scorpaena), the stonefish (Synanceja), and the lionfish (Pterois), and because he believes these venoms are chemically similar, this hypothesis may also be valid for the lionfish.

CONCLUSIONS

The fact that lionfish venom adversely affects the cardiovascular system of another marine teleost cannot be questioned. It is still not known, however, whether these adverse effects of the venom are direct actions on the cardiovascular system, or indirect liberations of other substances induced by the venom. The effects produced by lionfish venom cannot really be explained by a single mechanism, or a single component of the venom, and future work with this toxin should be directed toward delineating the mechanism(s) of action.

Considering the relatively large number of venomous fish species in existence today, and the great potential for the biomedical use of these toxins (Baslow, 1977; Colwell, 1983; Der Marderosian, 1969; Ruggieri, 1953), it is astonishing how little is actually known about these venoms. As stated previously, the major reason for our lack of knowledge in this area is the extreme instability of most fish venom preparations. The successful long-term stabilization of lionfish venom with the use of protease inhibitors as introduced here, may have partially solved this research obstacle. Hopefully this technique may be applicable to the further study of this and of other fish venoms. Alternatively, Russell and Brodie (1974) have suggested that it may be possible that when a lethal fraction of fish venom is ever isolated to its purest form, it may be inherently stable without the addition of any chemicals.

Russell and Brodie (1974) also point out that a crude fish venom extract contains a number of equally complex components, in addition to the venom itself, each possessing differing pharmacological activities. They suggest that since the glandular venom producing tissues are often found associated with isolated groups of cells, rather than a true venom "gland," that these other cells may be involved in the protection of the animal from its own venom. These other components in a crude venom extract may modify or destroy the toxic component. Russell states that it is ecologically valid that these fishes might possess some built-in protection against their own defensive venom.

The present work is only the second controlled pharmacological investigation of lionfish venom and this is by no means the final word. Much of the work in this thesis is, admittedly, preliminary. More work needs to be done in both the pharmacological and biochemical areas of venom research. Specifically, experiments need to be directed toward delineating the venom's mechanism(s) of action, including evaluating the possible effects on other systems such as the neuromuscular junction. Additionally, the venom and/or its components need to be purified to a much greater degree, ultimately leading to the determination of a chemical structure.

In closing, I would like to point out that Russell has stressed, in his proposed protocol for the analysis of fish venoms (Russell and Brodie, 1974), the importance of "survey preparations," such as the preparations used in this study, "to gain insight into the cardiovascular effects of a toxin." Finally, Russell has also

concluded that, despite the obstacles, fish venom research should continue:

> "Future work should also be directed toward improving our isolation techniques to the point where some of the marine toxins can be characterized as pure products and then synthesized for use as tools in molecular biology or as drugs for mankind. No other area of biology has a greater potential for the development of useful experimental and medical products as does that of marine toxins."

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APPENDIX

APPENDIX

1) UNSUCCESSFUL VENOM EXTRACTION TECHNIQUES

Three methods of venom extraction, which did not involve the killing of the animal, but merely "milking" it of its venom, were attempted without success. The methods used were modifications of various live animal venom extraction methods, used successfully by other researchers with species of venomous fishes other than the lionfish.

A) Rubber Sheet Method

The rubber sheet method, for live animal venom extraction, was developed by Endean (1961) who studied the venom of the stonefish, *Synanceja trachynis*. His technique involved pulling a rectangular rubber sheet (2 mm thick) down over each individual dorsal spine. The rubber sheet was easily pierced by the rather strong, sharp dorsal spines of this species, and the sheet was pushed towards the proximal end of each spine. Stonefish venom was "violently expelled" from the twin venom ducts after the surrounding integumentary sheath had first constricted and then stretched the venom glands. Approximately 0.1 ml of crude venom was obtained in this manner, from each spine, which was then easily pooled and collected. Endean found that this species of stonefish could regenerate its venom glandular contents in three weeks time, demonstrating that some venomous fishes could indeed be "milked" of their venom without harm. Cameron and Endean

(1966) also used this technique successfully with the scorpionfish, Notesthes robusta.

A modification of this method was attempted with the lionfish in this study. Rubber sheets similar to those used by Endean, dental dam material, prophylactic rubber material, and parafilm were all used in an attempt to atraumatically extract the lionfish venom. None of these materials, however, were able to be gently pierced by the extremely thin, and somewhat fragile, dorsal spines of the lionfish without causing substantial damage to the animals. Considerable contamination from various adjacent cellular constituents was also present in the resultant extracts. Additionally, the venom gland morphology of the lionfish is considerably different from that of the stonefish (see text). Lionfishes do not possess venom "ducts" as do the stonefish, which may help to explain why this method of venom expulsion was not successful.

B) Hypodermic Needle Method

This method of venom extraction, developed by Saunders and Tokes (1961), involved puncturing the bilateral venom sacs (two per spine) of the stonefish, Synanceja horrida. After puncturing the sacs, the free-flowing venom could be easily collected into vials. Saunders and Tokes were able to obtain approximately 0.2 ml of crude venom per fish, and regeneration of the sacs was possible.

This method was also attempted with the lionfish, but resulted in extreme cellular contamination, and no toxic activity in the crude extracts. This method also severely damaged the animals.

C) Sponge Method

The sponge method of Carlisle (1962) involved placing a weeverfish (*Trachynis vipera*) in a small tank of seawater, and then provoking it with a small piece (6x6x6 mm) of polyurethane sponge, held in forceps, until the animal elicited its defense response and stabbed the sponge. After the stabbing, the sponge was held against the fish for 15 seconds, and then removed and washed three times with 1.0 ml volumes of distilled water. Carlisle found this method to give an adequate yield in the weeverfish, and venom gland regeneration was apparent in as little as three days.

This method of venom extraction was also attempted with the lionfish, unsuccessfully. Again, due to the morphology of the lionfish venom apparatus, and the diffuse nature of the venom glands, this method resulted in a high degree of contamination from extraneous tissues, dilution, and the resultant extract possessed no activity.

It was after these multiple unsuccessful attempts that the present method of venom extraction, via direct venom apparatus removal and homogenization, was chosen.

2) <u>MARINE ANIMAL GELATIN DIET</u>

Gelatin diets, for the nutritionally sound feeding of marine fishes and invertebrates, are not a new approach. Extensive research has been done to determine the nutritional requirements of some of the most commercially important species of fishes (i.e. trout, salmon, catfish) including but not limited to the amino acid, protein, lipid, carbohydrate, and vitamin requirements. These values, although not exact, have been used successfully with other species of fishes.

A gelatin diet is merely a "shotgun" method of maximizing these values in a single formula. In diet design, some consideration is given to the type of fish and its mode of life, and the constituents are adjusted accordingly. Other considerations include: 1) using only ingredients that are able to be assimilated by the fish (i.e. fish flesh instead of beef), and 2) adjusting the physical consistency to allow the diet to either float or sink, depending on the needs, and also to prevent its breakdown in water.

The advantages of a gelatin diet are numerous. It saves the trouble of having to vary single diets on a regular basis to achieve a nutritionally balanced diet; it is quite easy to prepare, store and serve, and at a much reduced cost; and it is essentially nutritionally complete in itself.

The recipe for the diet which was fed to the buffalo sculpin in this study is as follows:

1) Thaw all ingredients first (in hot water).

2) Place the following ingredients into a one gallon commercial blender:

A)	Oregon Moist Pellet (OMP)	30 oz.
B)	Smelt or Herring	20 oz.
C)	Squid	10 oz.
D)	Shrimp	10 oz.
E)	Spinach (frozen)	10 oz.
F)	Carrots (frozen)	10 oz.
G)	Aminoplex Solution (or AA 1000)	4 cc
H)	Paprika	4 Tbl.
I)	<u>HOT</u> water (from tap)	2200 ml

3) Blend on low setting for 30 seconds (no longer), and then on medium for 30 seconds.

4) Add one package of gelatin (16 oz.) in three aliquots, blending well after each addition.

5) Pour into four tupperware (freezer) containers.

6) Put containers into refrigerator or cold room until gelatin sets up (2-6 hours).

7) Put into freezer.

3) VASCULAR CANNULATION PROCEDURE

Introduction

In the many aspects of fish physiological research (e.g. chemistry, pharmacology, nutrition, toxicology, cardiovascular physiology) there is often the need to continuously sample and monitor the cardiovascular system. The development of techniques for the vascular cannulation of fishes (eg. Smith and Bell 1964, 1967) has made it possible to investigate the various problems of cardiovascular function and haematological effects in fishes. Most of the earlier methods involved the use of hypodermic needles to introduce the polyethylene catheters into the vasculature. Problems arise with these methods when the needle, even if ground blunt, rubs against the vessel walls causing contralateral damage which can provide the means for blood clot formation.

We have developed a new and relatively simple method of dorsal and ventral aortic cannulation, via the branchial arteries, that offers maximal experimental versatility. Our method allows for: 1) multiple cannulation of the dorsal and ventral aortas for comparative studies of the arterial and venous systems, 2) easy, rapid, and multiple blood sampling, 3) injection of drugs or xenobiotics, and 4) the monitoring of blood pressure and heart rate.

Methods

Buffalo sculpin (*Enophrys bison*) were chosen as the experimental animal for this study because of their local abundance and easy

maintenance. The buffalo sculpin is a eurythermal, euryhaline, benthic cottid which serves as an excellent test organism for surgeries due to its inactive behavior and durability. Adult sculpin (250-900g) were collected by otter trawl from Yaquina Bay, Newport, Oregon, and were maintained in 150 l aerated, running seawater tanks at 12°C for at least two weeks prior to surgery.

Fish were anesthetized with a seawater solution of tricaine methanesulfonate (MS 222; Sigma; 70 mg/l) until a point where the fish's righting ability was lost. Anesthetized fish were transferred and secured laterally to the trough of a specialized surgery table (modified from Smith and Bell 1967). This surgery platform was equipped with recirculating seawater of the same anesthetic solution for continuous gill irrigation. The left operculum of a sculpin was retracted with a hemostat attached to the table to expose the gill arches. The second, third, or fourth gill arch was first isolated by dorsal and ventral ligatures which were of sufficient tension to stop blood flow in the branchial arteries, but not enough to sever the arch. Thick carpet thread was used here, rather than silk or nylon suture, to prevent damage from cutting. The gill filaments of the isolated arch were trimmed away between the knots to expose the inside base of the gill arch. Removal of these filaments from a few of the gill arches does not seem to adversely affect oxygen uptake of this species, nor has it been shown to affect other species of fish. A V-shaped notch is then cut into the base of the gill arch, exposing the afferent and efferent branchial arteries.

Cannulae consisted of 40 cm lengths of polyethylene tubing (PE 50; 0.58 mm x 0.96 mm i.d.; Intramedic) which were filled with 0.2 ml (cannula void volume) of 500 I.U./ml heparinized (Sigma) marine teleost saline (Stuart et al. 1983). A 23 gauge needle and a 5 cc syringe were attached to one end of the cannulae, while the other end was cut to form a taper to allow an easy entry into the branchial vasculature.

Cannulae were inserted either ventrally into the afferent branchial artery toward the ventral aorta, or dorsally into the efferent branchial artery toward the dorsal aorta, or both. Additional force must be applied to to the cannulae to force them through the vasculature ligatures around the gill arch. Once beyond this point, the cannulae were positioned back and forth until blood can be drawn into the cannulae with a syringe. Correctly implanted cannulae were then secured to the gill arch with three sutures, and then passed out of the opercular cavity along the dorsal or ventral areas of the body. To prevent the fish from damaging or snagging the cannulae, each cannula was secured along the body with cutaneous sutures (4-0, Ethicon).

Fresh seawater is exchanged for the seawater-anesthetic solution irrigating the fish's gills on the surgery table until opercular movements are observed. Cannulated fish were then transferred back to their anesthetic-free seawater aquaria for recovery. If the animals were to be used for cardiovascular studies involving the monitoring of heart rate and blood pressure, they were first secured in special plexiglass restraining cages

before recovery. Those animals cannulated only for the purpose of blood sampling were allowed to swim freely, towing their connected syringe at the water surface.

Most animals were used for cardiovascular studies by coupling the end of one of the cannulae of each animal to a pressure transducer (Gould-Statham P23De) interfaced with a transducer amplifier (Gould 11-4307-04) and a chart recorder (Gould-Clevite Brush Mark 260). The monitoring of the cardiovascular parameters was usually begun immediately following surgery, however, animals were allowed to recover for 24 hours before perturbation.