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THOMSON, Donald Arthur, 1932—
A HISTOLOGICAL STUDY AND BIOASSAY OF THE TOXIC STRESS SECRETION OF THE BOX-FISH, OSTRACION LENTIGINOSUS.

University of Hawaii, Ph.D., 1963
Zoology

University Microfilms, Inc., Ann Arbor, Michigan
A HISTOLOGICAL STUDY AND BIOASSAY OF
THE TOXIC STRESS SECRETION OF
THE BOXFISH, OSTRACION LENTIGINOSUS

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN ZOOLOGY

JUNE 1963

By
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PLEASE NOTE: Tables and Figures are not original copy. Some of these pages tend to "curl". Filmed in the best possible way.

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The adult boxfish, *Ostracion lentiginosus*

male: top
female: bottom
PREFACE

A fire which leveled one of the buildings of the Hawaii Marine Laboratory on Coconut Island, December 30, 1961, destroyed all the original notes and data pertaining to this thesis collected since 1959. Therefore, some of the information in Chapters 5 through 13 is based upon progress reports submitted at the end of each school semester to Dr. Pieter B. van Weel, Chairman of the Thesis Committee. The important experiments in these chapters were repeated and the entire histological investigation was conducted after the fire.

The University of Hawaii, through the intercession of the Dean of the Graduate School, Dr. Robert W. Piatt, awarded me a one-year grant to compensate for losses suffered in the fire and to enable me to complete this thesis. For this I am very grateful. I would also like to thank Drs. Albert H. Banner and Philip Helfrich of the Hawaii Marine Laboratory for their interest in this investigation and their attempts to procure funds for this study. I wish to give a special acknowledgement to Dr. van Weel for his foresight in requiring detailed progress reports, a measure which prevented more serious losses of data in the fire, and for his guidance and sound judgment during the course of this research. I am indebted to many students and faculty members, especially Charles B. Alender, William Eger, David Boylan, and Ralph T. Hinegardner for stimulating discussions which led to many new ideas on how to approach this problem.

I wish to thank Mr. John Marr, Director of the Honolulu Biological Laboratory, Bureau of Commercial Fisheries, and Mr. Vernon E. Brock, former Director, for their permission to use the docksite facilities at Kewalo Basin. Thanks also to Mr. Dick Holoway and his assistants of the same laboratory for providing tuna blood and saline solution.

I am greatly indebted to my wife, Jenean, for the graphs, fine illustrations and the typing of the manuscript, arduous tasks which I would not have accomplished without her help.
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ABSTRACT

An ichthyotoxin was isolated and partially purified from the foamy mucous secretions collected from the skin and mouth of distressed boxfish, *Ostracion lentiginosus*. The thermostable, non-dialyzable toxin foamed profusely in aqueous solutions and was highly toxic to fish and many other aquatic organisms at concentrations as low as 1.0 ppm. In addition, this toxin caused agglutination and hemolysis of vertebrate erythrocytes and formed a water-insoluble, non-toxic complex with cholesterol. Thus, the toxin secreted by the boxfish was tentatively identified as a steroid saponin and the name "ostracin" was proposed, marking the first known occurrence of saponins in vertebrates.

A histological investigation of the integument and mouth of boxfish revealed three types of gland cells: mucous cells in the superficial layers of the epidermis; so-called "club cells" in the intermediate and basal layers, characterized by mid-epidermal extrusion of their secretory products; and "labial cells" lining the labial villi and forming compound glands in the buccal cavity.

Developmental, or restitutional stages of the club cells were described and some staining properties of their homogenous and granular secretions were determined. Clusters of these cells formed primitive multicellular epidermal glands characteristic of those found in venomous fishes, and it was proposed that these club cell glands were the
source of ostracin secretion in the skin.

Ostracin could not be extracted from the skin of dead boxfish, although large amounts (100 mg of semi-pure toxin) could be readily collected in the mucous secretions of living, but distressed boxfish. Furthermore, ostracin was toxic to the boxfish itself and caused agglutination and hemolysis of boxfish erythrocytes in vitro, suggesting that ostracin was activated during, or just prior to, extrusion from the gland cell.

The ichthyotoxicity of the foamy oral mucus of distressed boxfish was attributed to secretions of the labial glands in the buccal cavity although these oral exudations did not contribute significantly to the total toxicity of the stress secretions. The chemical identity of the oral and skin toxins was not determined.

A comparative histological study of the cowfish, *Lactoria fornasini*, a closely related species that also secreted a hemolytic ichthyotoxin with its mucus, corroborated the results obtained with *O. lentiginosus*.
Poisonous fish have been considered as belonging to one of two categories: (1) fish that are only poisonous when eaten and (2) fish that have venomous spines or stings. The first category may be divided into two groups: fish with poisons believed to be of exogenous origin, i.e., the ciguatera toxin, which is of regional occurrence in the tropics, may be found in many widely divergent species of bony fish and is thought to be of environmental origin; and fish with poisons believed to be endogenous, i.e., the puffer-fishes which naturally contain toxins in their flesh, viscera and skin that are believed to be metabolically produced by the fish themselves. The functional significance of such toxins is unknown.

The second category includes all so-called venomous fishes such as the stonefish (*Synanceja*), weeverfish (*Trachinus*), or lionfish (*Pterois*) that have venom sacs at the base of their spines or venomous skin glands associated with spines. Such poisonous spines are clearly evolutionary specializations and are assumed to have defensive functions.

A possible third category of poisonous fishes, those that secrete substances into the water that are poisonous to other fish, has long been neglected.
Recently Clark and Gohar (1953) reported that a Red Sea boxfish (Trunkfish family: Ostraciontidae) seemed to secrete a poison into the water killing other fishes. The authors write:

"It is, however, common experience at the Biological station of Al Ghardaqa that it is dangerous to put boxfishes into small aquarium tanks containing other fishes, as this often leads to the death of many of the fish inhabitants of those aquaria. The boxfishes seem to pass some poisonous secretion into the water, but the exact nature of the problem still awaits investigation."

More recently Brock (1955) reported that the Hawaiian boxfish, Ostracion lentiginosus appeared to produce a substance poisonous to fishes. Brock noted that only boxfish under stress seemed to produce this poison as boxfish acclimatized to aquarium conditions did not poison the aquarium fish inhabitants. Only newly introduced, highly excited boxfish secreted this poisonous substance.

Tropical saltwater fish hobbyists have probably become aware of the poisonous character of boxfishes through experience. Straughan (1959) noted that the Atlantic spotted trunkfish (probably Lactophrys bicaudalis, author does not give species) apparently gave off a highly poisonous substance when disturbed and cautioned aquarists on the use of this fish in a marine aquarium.

Poisonous secretions by distressed fish apparently
are not confined to the trunkfishes. Whitley (1957) reports a similar phenomenon in a frogfish, a distantly related species (family Batrachoididae; frogfish = toadfish):

"Sir Edward Hallstrom has informed me that a local frogfish is exhibited at Taronga Park Aquarium, Sydney, where they have also had specimens of a more ornamental New Guinea species; but these fishes squirt out a liquid or slime which fouls the water and kills other fish. Some species have a pore in the pectoral axil or 'armpit', the function of which is unknown, though it resembles the poisonous gland of catfishes."

Some puffer-fishes, fish closely related to trunkfishes, apparently produce toxic stress secretions. During preliminary investigations of boxfish secretions, it was accidently discovered that the mucous secretions of a puffer-fish, *Arothron hispidus* (family Tetraodontidae) were highly toxic when injected into fish and mice. However, unlike boxfish secretions, puffer secretions were not poisonous to fish immersed in sea water containing these secretions.

Further investigation showed that two other puffers, *Diodon hystrix*; (family Diodontidae) and *Canthigaster rivulatus* (family Canthigasteridae) also produced mucous secretions that had a similar toxic effect as those of *A. hispidus*. It should be emphasized that these puffers contain a potent endotoxin in their flesh, viscera, and skin and the relationship between this
endotoxin and the toxic mucous secretions requires clarification.

The fact that fish under stress produce substances that are not produced under normal conditions has long been recognized, although such secretions are not necessarily toxic. Von Frisch (1941) showed that in minnows an alarm reaction was induced by substances produced by distressed minnows or an extract of minnow skins. Furthermore, Skinner et al. (1962) and Verheijen (1962) reported species-specific alarm substances from the top smelt (Atherinops affinis) and the freshwater creek chub (Semotilus atromaculatus). More recently Tester (1963) found that hungry sharks could detect a scent released by alarmed but uninjured prey fish and postulated that distressed fish produced a substance attractant to sharks.

The trunkfishes, because of their toxic stress secretions, provide extreme examples of the production of stress substances by highly excited fish, and this toxicity has possibly caused these fish to be included in general literature on poisonous fishes. For example, Randall (1958), in a review of ciguatera poisoning, mentions that Ostracion is poisonous and also gives a reference (Brown 1945) to a toxic Atlantic trunkfish (believed to be Lactophrys bicaudalis)
which states:

"... the poison is localized in small pockets of jelly on each side within the carapace just behind the gill opening. Market vendors in Nassau cut this out before selling the fish: symptoms are unsteadiness in gait similar to drunkenness (Clarke 1920) and the effects may be serious." (page 36).

In another instance Halstead and Bunker (1954) noted that muscle and visceral extracts of *Ostracion meleagris* (synonym of *O. lentiginosus*) and *O. cubicus* from Johnston Island, a ciguatera toxic area, were toxic to mice, however it was not clear whether the toxic effects were attributed to a ciguatera poison or a special trunkfish toxin.

Unfortunately the literature on poisonous trunkfish is sparse and sketchy, and no systematic investigation has been conducted on trunkfish toxins.

The purpose of this thesis is to investigate the phenomenon of toxic stress secretions by the boxfish, *Ostracion lentiginosus*. Bloch and Schneider, the most common trunkfish in Hawaiian waters and the species that Brock (1955) had reported as producing a poisonous substance. The aims of this investigation are: (1) to determine the effect of the boxfish toxin on various living systems in order to develop a bioassay for the toxin, (2) to gain information on the probable origin of the toxin, i.e., exogenous or endogenous, (3) to isolate and partially purify the toxin contained in
the stress secretions in order to (4) determine some of the toxin's physical properties and learn its general chemical nature, (5) to conduct a thorough descriptive histological study to determine the site of secretion of the toxin and the probable gland cells, (6) to compare the toxic stress secretions of the cowfish, *Lactoria fornasini* Bianconi, to those of the boxfish with respect to the general nature of the secretions and the histology of the secretory tissues, to obtain a broader understanding of toxic stress secretions by trunkfish.
CHAPTER 2
SYNOPSIS OF THE FAMILY OSTRACIONTIDAE:
SYSTEMATIC AND ECOLOGICAL CONSIDERATIONS

Fishes of the family Ostraciontidae, commonly called trunkfishes, are readily identified by their rigid dermal carapace consisting of firmly united polygonal bony plates. Conspicuous fleshy lips, slit-like gill openings, flexible pectoral, dorsal and anal fins, and a muscular caudal peduncle with a rudder-like caudal fin complete the identification for the most casual observer.

The systematic position of the trunkfishes is in the order Tetraodontiformes (Plectognathi) which includes such highly specialized and bizarre forms as the puffers, filefishes, triggerfishes and the ocean sunfishes. Superficially, these fishes do not resemble one another except that all have neither normal pelvic fins nor normal scales and all have evolved an assortment of curious protective devices. The basis for classifying the plectognaths is the similarity of osteological characteristics which indicates that this group was derived from an acanthuroid-like fish.

The systematics of the family Ostraciontidae is somewhat confused. Fraser-Brunner (1935) lists 12 genera under two subfamilies, using the 5 longitudinal ridges of the carapace as key characteristics in separating the
genera. In a later paper the same author (1941) separates the subfamilies into two distinct families: Aracanidae and Ostraciontidae, while Gosline and Brock (1960) prefer to include all trunkfishes under a single family, Ostraciontidae. At present there seems to be need for a revision of the family.

ECOLOGY OF TRUNKFISH

The trunkfishes, like most of the plectognaths, are coral reef fishes, inhabiting the shallow inshore waters of tropical seas. They are circumtropical in distribution and their pelagic larvae are frequently collected in surface plankton tows many miles from shore. The adults, with a few exceptions, are closely associated with a substrate.

The feeding habits of trunkfish are poorly known. Hiatt and Strasburg (1960) in a study of the ecology of Marshall Island fishes examined the gut contents of 6 specimens of Ostracion cubicus in Eniwetok and stated that trunkfish are completely omnivorous, taking what food items they can get. However, the authors recognized the trunkfish's essentially herbivorous nature when they placed them in the category of grazers. They further considered all the Marshallese plectognaths, which include triggerfish, filefish, trunkfish, and two families of puffers, as facultative omnivores, i.e., fish capable of taking full meals of animal or plant matter, depending on which appears to be most available.
PROTECTIVE ADAPTATIONS OF PLECTOGNATHS IN GENERAL

The most striking characteristics of the plectognaths and most pertinent to this study are the diverse protective adaptations that have evolved in this group. The best known of these is the ability of the puffers, also known as balloon fish, blowfish etc., to inflate with either air or water, thus effectively increasing their size and discouraging predators. The well known toxic quality of their flesh may also serve as a deterrent to predation. The filefishes and triggerfishes have developed a rigid dorsal spine which locks in an upright position when these fish are threatened, while the trunkfishes have a tough bony carapace which provides a durable protective armor. Besides these devices, the puffers and trunkfishes have been recently found to secrete "toxic mucus", the elucidation of the trunkfish secretion being the theme of the present investigation.

The value of such peculiar defensive adaptations to the plectognaths is better appreciated when one considers their poor swimming abilities, making them easy prey for fast-swimming fishes. Although juvenile plectognaths are commonly found in the stomachs of yellowfin tuna (Reintjes and King, 1953), this is not disconcerting when we consider the relative sizes of prey to predator, whereupon a protective device of almost any sort would be functionally useless. In their normal habitat on the reef where large,
fast-moving predators like the tuna are replaced by smaller, more deliberate stalkers like the barracuda, carangids and moray eels, the plectognaths have the advantages of both an access to shelter and their unusual protective devices.

Although it is indeed difficult to ascertain the survival value of an adaptation, it has long been recognized (for example see Dobzhansky, 1951) that if a modification benefited an organism in only one instance out of a thousand it would have sufficient survival value to the species as a whole to be genetically selected in subsequent generations.
CHAPTER 3

THE HAWAIIAN TRUNKFISHES

The Hawaiian trunkfish fauna is represented by 3 genera and 5 species according to Gosline and Brock (1960). Only 2 species are common to inshore waters: *Ostracion lentiginosus* and *Lactoria fornasini*. *Lactoria diaphanus* is more frequently encountered in offshore waters and is essentially a pelagic species. *Ostracion solorensis* is quite rare, being known only from a single Johnston Island specimen and *Aracana aculeata* is known from Hawaiian waters from two specimens dredged at a depth of 500 feet. Another species, *Rhynchostracion (?)* was collected over deep water off Pokai Bay, Wainae, Oahu during the course of this investigation.

The most common species of trunkfish around the island of Oahu, and perhaps throughout the Hawaiian Islands, is the boxfish, *Ostracion lentiginosus* Bloch and Schneider, which is the species most thoroughly studied in the present investigation. There is some disagreement concerning the nomenclature of this species. Briggs (1962) states that *O. lentiginosus* is a synonym of *O. meleagris* Shaw, basing his decision on the work of Rofen (1958). Rofen (1958) states that he follows both Fraser-Brunner (1935) and Schultz (MS). Since *O. lentiginosus* Bloch and Schneider (1801) appears to have priority over *O. meleagris* Shaw (1804),
the former name will be used in this investigation.

Sexual dimorphism is frequently encountered among the trunkfishes. There are obvious, distinct differences between males and females of *O. lentiginosus*. The male boxfish was originally described as a separate species, *O. sebae* Bleeker, and only the female was known as *O. lentiginosus*, but Fraser-Brunner (1940) established that *O. sebae* was indeed the male of *O. lentiginosus*. The male is distinguished by its convex snout without spots, ocelli along the sides of the body and along the ventral carapace, and by a conspicuous golden yellow interorbital band. Juveniles and females have a slightly concave snout with spots, uniform white spots over a dark blue background covering the entire body, no ocelli and no yellow band between the eyes (see Frontispiece and Figure 1).

There is no apparent sexual dimorphism in the other common Hawaiian trunkfish, *Lactoria fornasini*. This species has a pale brownish carapace sparsely marked with iridescent blue spots. The protruding horns extending forward from the eyes have caused this species to be called "cowfish".

Literature on trunkfishes is very sparse, especially with respect to general biology and life history. Most references to trunkfishes have been concerned with systematics or morphology. For example, Clark (1949) gave a key to the Hawaiian trunkfishes along with general notes on some species, and Rosen (1912-1913) included trunkfish in
his studies on the plectognaths, a series of four papers dealing with the blood vascular system, the air sac, the integument, and the body muscles. Al-Hussaini (1947) briefly described the feeding habits and morphology of the alimentary tract of a Red Sea trunkfish and Willem (1941) described the function of the respiratory apparatus of *O. cornutus*. The major taxonomic works have been done by Fraser-Brunner (1935, 1941).

References to the possible production of a poisonous substance by trunkfish have already been cited but these were merely brief notes commenting on the apparent poisoning of other fishes by boxfish in aquaria.

An often quoted observation on trunkfish locomotion by Goode (Evermann, 1902) is typical of the kind of information available:

"The locomotion of the trunkfishes is very peculiar. The propelling force is exerted by the dorsal and anal fins, which have a half-rotary, sculling motion, resembling that of a screw propeller; the caudal fin acts as a rudder, save when it is needed for unusually rapid swimming, when it is used as in other fishes; the chief function of the broad pectorals seems to be that of forming a current of water through the gills, thus aiding respiration, which would otherwise be difficult on account of the narrowness and inflexibility of the brachial apertures. When taken from the water one of these fishes will live for two or three hours, all the time solemnly fanning its gills, and when restored to its native element seems none the worse for its experience, except that on account of the air absorbed, it cannot at once sink to the bottom." (p. 261)

The last sentence of the above quotation was certainly not true for Hawaiian trunkfishes. Boxfish and cowfish
became moribund if left out of water longer than ten minutes and the fanning of the pectoral fins appeared to be related to the secretion of toxin rather than an emergency respiratory aid as the above quotation seems to suggest.
CHAPTER 4

COLLECTION AND PREPARATIVE TECHNIQUES

COLLECTION OF TRUNKFISH

Nearly all boxfish used in this study were captured in Kaneohe Bay, the Ala Moana reef and Kewalo Basin, Oahu. The major collecting site was Kewalo Basin, a harbor for small fishing vessels. The actual collecting occurred on the seaward side of the basin opposite the Fish and Wildlife Service Laboratory along the boat piers and along the stone wall parallel to the seaward channel. Here the boxfish could be captured by traps or dipnets. Along the Ala Moana reef adjacent to Kewalo Basin, boxfish and cowfish were captured only with dipnets. The largest boxfish were collected by fish traps in Kaneohe Bay.

Trapping with commercial fish traps constructed of iron frames and 1-inch chicken wire proved moderately successful in capturing adult boxfish and large cowfish, although difficulties were met when traps were set in the shallow water preferred by these trunkfishes as traps were inevitably stolen or tampered with by curious fishermen or skindivers. Traps set in water sufficiently deep to prevent the trap from being seen from the surface usually caught few boxfish and were difficult to locate and haul. The traps set in the shallow waters opposite
the government biological laboratories in Kewalo Basin were relatively safe from intruders during the working week but during weekends had to be lifted and stored on land.

The problems of trapfishing made it necessary to capture the majority of boxfish by dipnetting. There was a definite advantage in capturing boxfish this way. Trap-captured fish usually had been confined in the trap for several hours or days before being removed. As a rule, under such conditions of prolonged stress the highly excited boxfish lost much of their toxic secretions, especially if the fish had injured themselves by swimming against the wire walls of the trap. Boxfish caught with a dipnet were always in better condition and the stress of capture could be minimized by a skillful pursuer. For these reasons it is important to discuss methods of stalking and netting boxfish.

Boxfish were often encountered around coral mounds in small aggregations of from 2 to 5 fish. When approached by a skin diver, the boxfish would often watch the diver cautiously, allow him to swim within a few feet, then dart into a hole or under a ledge. Unlike many reef fishes, the boxfish would not remain hidden for more than a few seconds and would frequently depart via a back exit. It was more difficult to net boxfish among coral mounds in water deeper than 5 feet, therefore it was
often necessary to maneuver the boxfish toward more shallow water, and if possible, away from shelter. If the diver succeeded in doing this, he could chase the boxfish toward shore where it could be trapped and scooped up easily with the aid of two dipnets. Often the boxfish would inadvertently "beach" itself in its frantic efforts to escape. Although catching boxfish in this manner was quite laborious, and weather and tidal conditions had to be favorable, the method was fairly efficient.

Juveniles (under 100 mm total length) were easily captured by the method described but adults were usually more wary and less easily tricked. The large females were especially difficult to catch. The males were less cautious, even appearing curious, and were sometimes as easily captured as the juveniles.

Cowfish, *L. fornasini*, were not as common as the boxfish. They were much slower and more awkward swimmers than boxfish and were very easily captured with dipnets, even in deeper water. Cowfish were commonly seen on the reef flats at night with the aid of gas lanterns and could be easily scooped up with dipnets. Boxfish, on the other hand, were rarely seen on the reef at night at which time they apparently seek shelter like most diurnal reef fishes.
COLLECTING BOXFISH MUCOUS SECRETIONS*

When newly captured boxfish were removed from the water and held in the hand or placed in a container, they exhibited a rather peculiar behavior. Besides flopping about like most fish out of water, boxfish moved their fleshy lips and exuded a foamy mucus from their mouths and, while apparently fanning their gills with a vigorous fluttering of the pectoral fins, they were exuding more foamy, soap-like mucus at the base of these fins. The anal and dorsal fins also fluttered vigorously and a soapy mucous secretion also exuded from the skin folds at the base of these fins. At the junction of the caudal peduncle with the carapace more foamy mucus appeared when the tail was rapidly moved from side to side. By putting the boxfish into a beaker with a small amount of water and swishing the fish about for a few seconds, a very foamy solution formed (see Figures 2 and 3).

The procedure for collecting boxfish mucous secretions was to place freshly captured boxfish in separate small containers (plastic one-pint sherbet dishes or various sized glass jars) with a small amount of water (less than 50 ml). The container was sealed with a lid and the boxfish was swirled around at intervals of about one minute to cause further excitation. The stressed boxfish was left

* "Mucous secretions", as used in this thesis, means all external secretions by distressed boxfish which are collected with the mucus, but are not necessarily produced by mucus glands.
Figure 1. Juvenile boxfish. Note the resemblance to the adult female.

Figure 2. The foamy mucous secretions of a distressed boxfish out of water.
Figure 3. Collection of the entire stress secretions of a female boxfish.
in the container for 5 - 10 minutes or until death, although most of the mucous secretions appeared to be given off within the first few minutes. When removed from the container, the boxfish was rinsed with distilled or sea water to collect the foamy mucus adhering to the surface of its skin. It appeared that a more concentrated mucus was obtained when an adequate volume of rinse water was used, since water bathing the skin seemed to facilitate mucous secretion. (Too great dilutions of the mucus were avoided since it was later found that the boxfish toxin was labile in greatly diluted aqueous solutions.)

The resultant diluted mucous secretions were a foamy, colloidal solution which, when dissolved in sea water, was poisonous to marine fishes at dilutions as great as 1:25,000. The ichthyotoxic substance(s) contained in the mucous secretion of _O. lentiginosus_ will be heretofore referred to as "ostracin", a tentative name derived from the genus _Ostracion_. The justification for using this name will be stated in a later chapter. *

* Through convention a compound is usually not named until an empirical formula is determined, however the distinctive properties of "ostracin" (which will be elucidated later) appear to justify the tentative naming of this toxin(s) without this information.
PRELIMINARY ISOLATION OF OSTRACIN

The most efficient method of obtaining large amounts of ostracin was by collecting mucous secretions of boxfish under stress so that the water soluble ostracin in the mucus would be dissolved in an aqueous rinse of the entire boxfish. The resultant aqueous solution would form a very stable head of foam when shaken. The chief impurities in this colloidal mucous solution were inorganic sea salts, coagulated mucus and sometimes, fecal matter. The latter two impurities and some colloids were removed by filtration and subsequent centrifugation at 15,000 rpm, and salts were eliminated by dialysis. The clear non-dialyzable supernatant fluid retained its original toxicity and still foamed profusely when agitated. The total residue of an aliquot of this solution was determined by weighing the residue after evaporation. The weight of the aliquot residue expressed the concentration of impure ostracin in mg/ml of aqueous solution. This ostracin solution was refrigerated but often became cloudy upon standing and was clarified by further centrifugation or filtration with only slight loss in toxicity.

The above separation procedure was refined during the course of the investigation (see Chapter 11) and the ostracin so obtained will be referred to as "semi-pure" to distinguish it from the fresh, untreated secretions of boxfish containing "crude" ostracin.
CHAPTER 5

DEVELOPMENT OF A BIOASSAY FOR OSTRACIN

The development of a bioassay sensitive enough to detect minute amounts of biologically active substances is an important step toward isolating and studying unknown toxins. Toxins, being highly potent substances, can be readily detected by their action on living systems. The response of a living system to a toxin must then be converted to a quantitative expression which will accurately assay the toxicity of an unknown sample. Toxicity is defined as the total amount of toxic material in a sample while the potency of a toxin is the capacity of a given amount of pure toxin to elicit a response. A good bioassay should provide an accurate determination of the former and a reliable estimate of the latter.

Ostracin, the toxic, water soluble fraction of boxfish mucus secretions, has already been shown to be poisonous to fish and in this sense resembles holothurin, the sea cucumber toxin (Nigrelli and Jakowska, 1960) and some red tide toxins (Abbott and Ballantine, 1957; Shilo and Rosenberg, 1960; Gates and Wilson, 1960). Both holothurin and the red tide toxins have also been reported to exhibit a variety of effects on diverse organisms from protozoans to mammals. Because of the ichthyotoxic parallelism of ostracin with holothurin and the red tide toxins, it was decided to
test ostracin on various aquatic organisms. This approach would not only facilitate the development of an adequate bioassay for ostracin but would provide comparative information on the mode of action of ostracin with respect to other marine biotoxins.

TOXICITY OF OSTRACIN TO AQUATIC ORGANISMS

Various species of aquatic animals were placed in sea water containing crude ostracin in concentrations which were highly toxic to marine fish, i.e., caused death of such fish within 10 minutes. The animals used were those that were readily available and the list of species tested represents a more or less fortuitous selection of phyla.

Since the various animals tested fell into three general groups, the reaction groups proposed by Abbott and Ballantine (1957) for the Gymnodinium toxin were used and are as follows:

(A) **Not affected.** Animals of this group showed normal behavior during immersion in ostracin solutions and survived when returned to fresh sea water. Polychaetes, colonial ascidians, some mollusks and most crustaceans belonged to this group.

(B) **Slowly affected.** In this category were included animals which showed various symptoms of poisoning while immersed in ostracin-sea water for several minutes to hours but usually recovered when removed and returned to
Fresh sea water. Anemones and sea urchins are examples.

(C) Rapidly killed. This last category included all the animals which were killed within minutes after exposure to ostracin. Fish and cephalopods fell into this group.

A summary of reactions of various species is found in Table I. The results summarized in this table compare favorably with those obtained by Abbott and Ballantine (1957) using toxic cultures of Gymnodinium veneficum, a red tide dinoflagellate. These authors concluded that the rate of penetration of the Gymnodinium toxin into the test organisms was the critical factor in delineating between groups B and C. This explanation appears feasible in regard to ostracin since many interesting parallels with the Gymnodinium toxin were observed.

A description of the symptoms of animals of groups B and C is given below.

COELENTERATA

Sea anemones often retracted their tentacles slightly and wound the distal ends into tight spirals. The swimming anemone Nectothelia lilae, however, relaxed its tentacles which subsequently became insensitive to probing. The polyps of the colonial hydroid Pennaria tiarella also became insensitive to probing whereas the control animals would immediately contract when touched with a dissecting needle. All these coelenterates would recover if placed in fresh sea water before they became moribund in the ostracin.
### TABLE I. TOXICITY OF OSTRACIN TO SOME AQUATIC ANIMALS

<table>
<thead>
<tr>
<th>Animal</th>
<th>Reaction Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COELENTERATA</strong></td>
<td></td>
</tr>
<tr>
<td><em>Anemonia mutabilis</em> (anemone)</td>
<td>B</td>
</tr>
<tr>
<td><em>Nectothelia lilae</em> (anemone)</td>
<td>B</td>
</tr>
<tr>
<td><em>Pennaria tiarella</em> (hydroid)</td>
<td>B</td>
</tr>
<tr>
<td><strong>PLATYHELMINTHES</strong></td>
<td></td>
</tr>
<tr>
<td><em>Dugesia</em> sp. (planaria)</td>
<td>B</td>
</tr>
<tr>
<td><strong>ANNELIDA</strong></td>
<td></td>
</tr>
<tr>
<td>Sabillid worms</td>
<td>A</td>
</tr>
<tr>
<td>Unidentified polychaetes</td>
<td>A</td>
</tr>
<tr>
<td>Parasitic marine leech</td>
<td>A</td>
</tr>
<tr>
<td><strong>MOLLUSCA</strong></td>
<td></td>
</tr>
<tr>
<td><em>Helcioniscus argentatus</em> (Opihi)</td>
<td>A</td>
</tr>
<tr>
<td><em>Polypus</em> sp. (octopus)</td>
<td>C</td>
</tr>
<tr>
<td><strong>ECHINODERMATA</strong></td>
<td></td>
</tr>
<tr>
<td><em>Echinometra mathaei</em> (sea urchin)</td>
<td>B</td>
</tr>
<tr>
<td><em>Echinometra oblonga</em> (sea urchin)</td>
<td>B</td>
</tr>
<tr>
<td><em>Echinothrix diadema</em> (sea urchin)</td>
<td>B</td>
</tr>
<tr>
<td><strong>ARTHROPODA: CRUSTACEA</strong></td>
<td></td>
</tr>
<tr>
<td><em>Spirontocaris marmoratus</em> (shrimp)</td>
<td>A</td>
</tr>
<tr>
<td><em>Stenopus hispidus</em> (banded shrimp)</td>
<td>A</td>
</tr>
<tr>
<td>Penaeid shrimp (prawn)</td>
<td>B</td>
</tr>
<tr>
<td>Pachygrapus sp. (crab)</td>
<td>A</td>
</tr>
<tr>
<td>Carphilus convexus (crab)</td>
<td>A</td>
</tr>
<tr>
<td><em>Artemia salina</em> (brine shrimp)</td>
<td>A</td>
</tr>
<tr>
<td><em>Labidocera</em> sp. (copepod)</td>
<td>B</td>
</tr>
<tr>
<td><strong>TUNICATA</strong></td>
<td></td>
</tr>
<tr>
<td><em>Ascidia</em> sp. (sea squirt)</td>
<td>A</td>
</tr>
<tr>
<td>Animal</td>
<td>Reaction Group</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>PISCES</strong></td>
<td></td>
</tr>
<tr>
<td>Mugil cephalus (mullet)</td>
<td>C</td>
</tr>
<tr>
<td>Mollienesia latipinna (sailfin mollie)</td>
<td>C</td>
</tr>
<tr>
<td>Limia vitatta (mosquitofish)</td>
<td>C</td>
</tr>
<tr>
<td>Gambusia affinis (mosquitofish)</td>
<td>C</td>
</tr>
<tr>
<td>Pranesus insolarum (iao)</td>
<td>C</td>
</tr>
<tr>
<td>Esoxus metallicus (minnow)</td>
<td>B</td>
</tr>
<tr>
<td>Tilapia mossambica (tilapia)</td>
<td>C</td>
</tr>
<tr>
<td>Abudefduf abdominalis (mao mao)</td>
<td>C</td>
</tr>
<tr>
<td>Acanthurus sandivicensis (manini)</td>
<td>C</td>
</tr>
<tr>
<td>Kuhlia sandvicensis (aholehole)</td>
<td>C</td>
</tr>
<tr>
<td>Chaetodon miliaris (butterfly fish)</td>
<td>C</td>
</tr>
<tr>
<td>Chaetodon lunula (butterfly fish)</td>
<td>C</td>
</tr>
<tr>
<td>Gymnothorax sp. (moray eel)</td>
<td>B</td>
</tr>
<tr>
<td>Carapus howei (pearlfish)</td>
<td>B</td>
</tr>
<tr>
<td>Ostracion lentiginosus (boxfish)</td>
<td>B</td>
</tr>
<tr>
<td>Lactoria fornasini (cowfish)</td>
<td>C</td>
</tr>
<tr>
<td>Arothron hispidus (puffer)</td>
<td>C</td>
</tr>
<tr>
<td>Canthigaster jactator (sharptailed puffer)</td>
<td>C</td>
</tr>
<tr>
<td>Canthigaster rivulatus (sharptailed puffer)</td>
<td>C</td>
</tr>
<tr>
<td>Bathygobius fuscus (gooy)</td>
<td>C</td>
</tr>
</tbody>
</table>
solution. The desensitization of the tentacles suggested a neurotoxic effect on the nerve-net system.

**PLATYHELMINTHES**

Planarians exposed to toxic solutions of ostracin in fresh water disintegrated upon transfer to clean water. Quaglia et al. (1957) reported similar results with holothurin-treated planarians. These authors also reported that planarians exposed to toxic holothurin solutions for less than one minute survived indefinitely, however when these animals were dissected transversely and returned to fresh water the anterior portions regenerated into complete animals, whereas the majority of the posterior portions disintegrated or failed to regenerate. A similar experiment was conducted in which 2 groups of 20 planaria (*Dugesia sp.*) were exposed to concentrations of semi-pure ostracin of 80 and 40 ppm (parts per million) for 15 - 25 and 5 - 15 minutes respectively. (These concentrations were highly toxic to fish.) The planarians were cut transversely while in the toxic solution and transferred to fresh water kept at room temperature (24 - 26°C). The anterior and posterior portions regenerated at the same rate as the controls. Eyespots were observed by the third day and the 40 ppm group appeared normal and survived as long as the controls. Although both portions regenerated equally well in the 80 ppm group, these animals were very inactive compared to
the control and 40 ppm groups. Many planarians were curled in a loop and were very "sticky" to touch. While the planarians in the control and 40 ppm groups were able to glide smoothly over the glass bottoms of their petri dishes, the 80 ppm group moved about by worm-like contractions. At the end of a month there was a 75% mortality rate in the 80 ppm group as compared to no mortalities in the 40 ppm and control groups. No anti-metabolic activity of ostracin was demonstrated in any group.

MOLLUSCA

An octopus (Polypus sp.) about one pound in weight was placed in a plastic bucket containing crude boxfish secretions. The animal clung tenaciously to the sides of the bucket and was pried loose with great difficulty. After about 5 minutes exposure to the toxic secretions the octopus was released in a large saltwater pond. The moribund octopus was found dead the next morning in the same spot where it had been released.

ECHINODERMATA

The tube feet of sea urchins contracted in ostracin solutions and the animals became immobilized, while control animals actively moved about in their containers. Injection of ostracin at the muscular base of the spine of the long spined species Echinotrix diadema caused rigidity of that spine but did not affect adjoining spines. Control saline
injections had no effect.

Ruggieri and Nigrelli (1960) found that holothurin was not only very toxic to sea urchin embryos but, in sublethal concentrations, caused abnormalities such as anisomalization and fragmentation. Because of the similarity of ostracin to holothurin, a series of experiments was conducted to determine the effect of low concentrations of ostracin on the developing embryos of *Tripneustes gratilla*. Although the data of these experiments were destroyed in the fire at the Coconut Island Marine Laboratory, December 30, 1961, a brief summary of results should be of some interest.

Fertilized eggs kept in sea water containing 1 to 10 ppm of ostracin showed marked inhibition of cleavage. In one experiment, eggs kept in sea water with 1 ppm ostracin developed only to the motile blastula stage while the controls developed into normal plutei during the same period. With a few exceptions, both in the controls and test animals, no abnormal larvae were observed.

Fertilized eggs of a sea cucumber, *Holothuria fuscorubra* were immersed in equivalent ichthyotoxic solutions of ostracin and holothurin (obtained from the cuvierian organs of *Actinopyga obesa*). Ostracin was lethal at 1 ppm while crude holothurin was non-toxic at this level but effective at only slightly higher concentrations (5 ppm).
CRUSTACEA

All crustaceans tested showed great resistance to ostracin. As suggested before, this may be due to the rate of penetration of ostracin through the gills or other body membranes. An inshore marine copepod, *Labidocera sp.*, normally a positively phototactic animal, i.e., one that is attracted toward light, lost its phototactic response in sea water containing ostracin and a 50% mortality ensued after 1½ hours. The control animals responded normally and survived the duration of the experiment.

PISCES

All fish immersed in ostracin-sea water were rapidly killed, usually within 10 minutes. The symptoms shown by all fish were identical and were very similar to symptoms reported by Abbott and Ballantine (1957) for the *Gymnodinium* toxin and by Nigrelli and Jakowska (1960) for holothurin. In ostracin concentrations causing death within 10 minutes (100 ppm), the initial reaction of fish when ostracin was added to the water was of marked irritability. The fish made violent attempts to swim away from the irritation and in some cases jumped out of the container. Such frantic swimming usually lasted 1 - 2 minutes, again depending upon the concentration of ostracin used. Opercular movements were erratic, spasmodic "vomiting" occurred, and
it appeared that the fish was desperately trying to expel water through its mouth. The fish then became quiescent and usually settled to the bottom of the container. The number of opercular movements, which determines the breathing rate, was greatly reduced. The fish then lost its balance control and could not right itself. Throughout this quiescent stage the fish sporadically darted aimlessly about the container crashing into its sides, and then slowly settled to the bottom. Just prior to death opercular movements were almost imperceptible and the moribund fish often made violent gasping movements and thrashed about. There was a gradual loss of sensory response preceding death with the base of the caudal peduncle retaining sensitivity longest. If the fish failed to respond when this area was pinched by a pair of forceps the fish was recorded as dead.

At a lower concentration (10 ppm) there was no violent initial reaction. Instead, the fish often made gasping movements at the surface of the water as fish normally do under low oxygen partial pressure. The other symptoms were identical to those described above.

**Effect of Body Size on Death Time**

The size of the fish appeared to have little effect on its survival in ostracin-sea water. Abbott and Ballantine (1957) found this to be true of gobies poisoned by the red
tide *Gymnodinium* toxin and explained this by the fact that
the toxin penetrates the gills and the active gill surface
is roughly proportional to body size within a species.

**The Critical Exposure Period of Fish to Ostracin**

The duration of exposure of fish to ostracin was critical.
Beyond a certain exposure time the effect was irreversible,
i.e., ostracin did irreparable damage and the fish did not
recover if returned to fresh sea water. Apparently a lethal
dose penetrated the fish very quickly or did morbid damage
to vital membranes like the gills.

An experiment was performed using juvenile tilapia,
*Tilapia mossambica* (15 - 20 mm total length), in which
groups of 4 fish were immersed in ostracin-sea water (about
5 ppm ostracin) for varying periods and returned to fresh
sea water for recovery. The results are summarized below:

<table>
<thead>
<tr>
<th>Exposure time (min.)</th>
<th>50% mortality (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>1½</td>
<td>36</td>
</tr>
<tr>
<td>1</td>
<td>...(1 fish died in 2 hours)</td>
</tr>
<tr>
<td>½</td>
<td>...(1 fish died in 2½ hours)</td>
</tr>
</tbody>
</table>

The tilapia exposed to ostracin for 30 seconds to 2
minutes showed no clear stress symptoms but became agitated
after 3 minutes and quiescent after 5 minutes.
Complete mortality ensued in all cases except at exposures of 30 seconds and 1 minute. These data suggested that ostracin either penetrated very rapidly or interfered with the surface of the gill membranes and that a lethal dose had been accumulated before outward symptoms were recognized.

**Response of Different Species to Ostracin**

The susceptibility of various species of fish to ostracin appeared to vary according to the activity level of different species. This can best be illustrated by relative survival times of different species immersed in equal concentrations of ostracin (about 5 ppm) in sea water:

(6 juvenile fish used per test; complete mortality occurred in all tests.)

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Survival time of 50% of fish (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abudedefuf abdominals</td>
<td>mao mao</td>
<td>6.0</td>
</tr>
<tr>
<td>Acanthurus sanvicensis</td>
<td>manini</td>
<td>8.5</td>
</tr>
<tr>
<td>Kuhlia sanvicensis</td>
<td>aholehole</td>
<td>10.0</td>
</tr>
<tr>
<td>Mugil cephalus</td>
<td>mullet</td>
<td>12.5</td>
</tr>
<tr>
<td>Mollienisia latipinna</td>
<td>sailfin molly</td>
<td>15.0</td>
</tr>
<tr>
<td>Bathygobius fuscius</td>
<td>goby</td>
<td>30.0</td>
</tr>
</tbody>
</table>

The first two species on the list are very active stenohaline reef fishes, the next three are moderately active euryhaline forms and the last, the goby, is a bottom dwelling, relatively inactive, tidepool species. Susceptibility to ostracin may be correlated with the frequency of opercular (respiratory) movements. The goby, typical of most bottom living fishes, has a much lower respiratory rate than active swimmers such
as the mao mao and manini and therefore, might be expected
to show a higher resistance to the toxin.

The Effect of Salinity on Toxic Action of Ostracin

The effect of salinity on the toxic action of ostracin
had a much more significant and dramatic effect on survival
rate than physiological variables. Juvenile tilapia acclimated
to sea water had a mean survival time of 20 minutes in
ostracin-sea water, but the euryhaline tilapia acclimated
to fresh water survived 70 minutes in the same concentration
of ostracin in fresh water. The freshwater cyprinid Esoxus
metallicus was even more resistant to ostracin in fresh water
than freshwater acclimated tilapia at the same concentration.
The mean survival time of these cyprinids was 3 hours and
50 minutes. Such anomalies led to the investigation of the
toxic action of crude ostracin on fish immersed in salinities
ranging from fresh water to sea water.

Juvenile sailfin mollies (12 - 15 mm total length)
were acclimated to water of various salinities ranging from
fresh to sea water for 25 hours. Groups of 6 fish were
placed in beakers containing 100 ml of the desired sea water
dilution. No aeration was used since it had been determined
that small mollies can survive without auxiliary aeration
for several hours to days under such conditions. A lethal
unit of freshly collected boxfish secretions was added to
each beaker. This experiment was repeated 3 times and the
results of the last 2 trials are depicted in Figure 4.
The data showed that there was a gradual increase in survival time of sailfin mollies as their sea water medium was diluted and at salinities ranging from 25 to 30% sea water there was a sharp increase in survival time. There was an equally sharp reduction in survival time in salinities below 25% sea water and in fresh water the survival time was only about twice as long as it was in sea water. The experiment was repeated with *Tilapia mossambica* and similar results were obtained.

The pH of the various dilutions ranged from 7.79 in 20% sea water to 8.04 in fresh water. The pH of the sea water used was 7.85. Although it seemed unlikely that the pH itself was exerting some effect on inhibiting the action of ostracin, an experiment was performed in which the pH of sea water was adjusted by adding acid or base. Groups of 4 tilapia (12 - 28 mm total length) were placed in beakers containing 400 ml of sea water of a pH ranging from 5.80 to 8.80. An equal amount of crude ostracin was introduced into each beaker and the mean survival time was recorded:

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean survival time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.80</td>
<td>24.0</td>
</tr>
<tr>
<td>6.35</td>
<td>25.0</td>
</tr>
<tr>
<td>6.97</td>
<td>24.0</td>
</tr>
<tr>
<td>7.70</td>
<td>23.0</td>
</tr>
<tr>
<td>8.40</td>
<td>21.0</td>
</tr>
<tr>
<td>8.80</td>
<td>22.0</td>
</tr>
</tbody>
</table>
Figure 4. Effect of crude ostracin on salpin mollies acclimated to different salinities.
It was clear that pH had little or no effect on survival times of tilapia immersed in ostracin-sea water.

Since sailfin mollies and tilapia are essentially freshwater fishes which have secondarily invaded brackish and salt water, it might be argued that these fish would be more resistant to ostracin while immersed in salinities more closely corresponding to their natural environment. Therefore, a more typically marine species, the aholehole (Kuhlia servicensis), was tested in a similar manner. This euryhaline species lives and spawns in sea water but the young are frequently encountered in brackish and freshwater streams. Juvenile aholeholes (25 - 40 mm total length) were acclimated for 24 hours to different salinities and 2 fish each were placed in beakers containing 100 ml of water of the desired salinity. A lethal unit of crude ostracin was put in each beaker. No aeration was used. The results are shown in Figure 5.

The mean survival time of the aholehole at low salinities was much longer than that of the sailfin molly and tilapia. Although this species was shown to be more susceptible to poisoning by ostracin in sea water than were sailfin mollies, the aholehole was found to be considerably more resistant to ostracin in dilute sea water than the molly was, especially in 20 to 40% sea water.

There are at least two possible explanations to this phenomenon. The first concerns the osmotic equilibrium of
Figure 5. Effect of crude ostracin on survival time of aholeholes (Kuhlia sanvicensis) acclimated to different salinities.
fish in changing salinities. It appeared that the rate of penetration of ostracin into the tissues of fish immersed in ostracin solutions was dependent upon the salinity of the medium. Osmotic regulation then was probably an important factor. According to Black (1957) the freezing point depression of the blood of freshwater teleosts is about -0.57° C and in marine teleosts is about -0.78° C, which is lower than that of the sea water (-1.8 to -2.2° C). Consequently, marine teleosts must actively take in water to replace water lost osmotically. The external membranes (gills, gut, skin) then favor water intake and are able to absorb water against an osmotic gradient. One can then expect a corresponding change in permeability and membrane potentials as marine fish are gradually acclimated to fresh water. The concentration of the blood of teleosts corresponds to 25 to 40% sea water, i.e., at this concentration the blood is isotonic with the external medium and osmotic work (exchange of water, salts and waste products) should be minimal. This coincides with the range of salinities in which ostracin was least effective and suggests that isotonicity is an important factor in the resistance of fish to ostracin.

The second possible explanation is that advanced by Yariv and Hestrin (1958) as reported by Shilo and Rosenberger (1960). The former workers, studying the ichthyotoxins from the phytoflagellate, *Prymnesium parvum*, have suggested
that a special property of the *P. parvum* ichthyotoxin was its requirement of cofactors for activity and that the toxicity of *P. parvum* solutions appeared to be a function of the concentration of both the ichthyotoxin and of inorganic salts. In the absence of inorganic salts, this ichthyotoxin does not kill fish.

The *P. parvum* ichthyotoxin has enough properties in common with ostracin to consider this cofactor theory as another possible explanation, except that ostracin, unlike the *P. parvum* toxin is more toxic in fresh water than in diluted sea water.

The important practical consideration here is that a change in salinity of the external medium will have a significant effect on toxicity of ostracin to euryhaline fishes and this fact should be taken into account in the development of a bioassay using fish immersed in water containing unknown amounts of ostracin.

**TOXICITY OF OSTRACIN TO THE PEARLFISH**

Pearlfishes, inquilines in the cloaca and respiratory cavities of certain sea cucumbers and starfishes, have been shown to be susceptible, but highly resistant to the sea cucumber poison, holothurin (Nigrelli, 1952) and *Carapus bermudensis* has been used for evaluating the toxicity of holothurin from the cuvierian organs of *Actinopyga agassizi* (Aronson and Mosher, 1951).
Pearlfish (*Carapus homeri*) were exposed to ichthyotoxic solutions of ostracin and crude holothurin (from skin of *Holothuria atra*) and their survival time recorded. Although the holothurin solution was more ichthyotoxic to sailfin mollies than the ostracin solution used (sailfin mollies died within 10 minutes in holothurin-sea water and in 15 minutes in ostracin-sea water), pearlfish lived for more than 6 hours in ostracin-sea water.

The pearlfish's resistance to both these toxins, and particularly to holothurin suggests some protective mechanism not possessed by other marine species (with exceptions in *Gymnothorax* and *Ostracion*).

**TOXICITY OF OSTRACIN TO THE BOXFISH**

Experience with collecting and transporting boxfish or cowfish showed that frequent mortalities occurred despite seemingly adequate aeration. This was especially true during transport of freshly captured trunkfish from which stress secretions had not been previously collected. Boxfish often became moribund during collection of their secretions if they were kept in the concentrated ostracin solution longer than 5 minutes. Cowfish appeared to be exceptionally sensitive to both their own and boxfish secretions. Thus, it appeared these trunkfish were being poisoned by their own toxins.

Experiments were performed to test this hypothesis.
Intramuscular and intraperitoneal injections of semi-pure ostracin in sea water media at doses of 3.0 to 7.0 mg/boxfish (80 to 117 mm long) did not cause death and immersion of boxfish in 15 ppm ostracin-seawater had no effect. However, when the crude mucous secretions of freshly captured boxfish were injected into the body cavity via the caudal-carapace juncture, the boxfish became moribund almost immediately, showing typical symptoms of ostracin poisoning.

About 1.0 ml of concentrated mucous secretions representing approximately one-quarter of the total stress secretions (equivalent to 20 - 30 mg of semi-pure ostracin) was needed to kill a 80 - 110 mm boxfish in 5 to 20 minutes. Lesser amounts were ineffective.

Thus, it is apparent that boxfish are susceptible to ostracin but are considerably more resistant to this toxin than other fishes.

TOXICITY OF OSTRACIN TO WHITE MICE

White mice have been widely used as assay animals for various biologically active compounds including plant and animal toxins. In studies on marine biotoxins white mice have been used as a standard assay for paralytic shellfish poisons, puffer toxins and ciguatera-type toxins and it has been customary to express the potency of any toxin by its LD$_{50}$ in white mice. (LD$_{50}$ is the minimum lethal dose that will kill just 50% of the assay animals.) Because of
the widespread use of white mice as indicators of toxin potency, it was necessary to determine the effect and potency of ostracin injected into mice.

**Symptoms**

Albino white mice (closed colony Carworth Farms Webster strain) ranging from 10g to 30g were injected intraperitoneally with crude and semi-pure ostracin. The characteristic symptoms of mice injected with ostracin are the following: about 2 - 3 minutes after injection the mouse appeared very sleepy and began to breathe deeply; this was soon followed by ataxia, the loss of its grasping and righting reflexes; breathing became more labored until the mouse appeared to be literally gasping for breath; "death" followed quickly, usually without convulsions. Autopsy revealed that the heart was still beating, the lungs were collapsed and, judging from the appearance of the peritoneum, there had been no vascular damage. If a sublethal dose was given, similar symptoms would often occur but recovery was usually complete within a few hours.

**Lethal Dose of Ostracin**

The lethal dose of ostracin in mice was quite high. At least 1 cc of a highly toxic 10 ml rinse was needed to kill a 20-gram mouse injected intraperitoneally. A dose of about 0.20 mg/g mouse of semi-pure ostracin appeared to be the minimum lethal dose. This amounted to about
4 mg per 20-gram mouse, a fairly large amount which certainly prohibited the use of mice as standard bioassay animals.

**Effect of Injection Media**

Since it was found that ostracin was less toxic to fish in fresh and 25 - 40% sea water than in 100% sea water, ostracin dissolved in distilled water and 25% sea water was injected intraperitoneally into mice to determine if similar reductions in toxicity occurred. Such injections caused no intoxication symptoms while sea water-ostracin injections of the same concentration were rapidly lethal. The cause of these anomalies is unknown, but may be explained by the same mechanism(s) suggested for ichthyotoxicity (see pp. 39 - 41).

**Control Injections**

Control injections of physiological saline, distilled water and sea water had no effect on mice. To determine if there was a toxic reaction to fish mucus in general, the mucous secretions of 4 species of reef fish were collected and assayed in the same manner as were boxfish secretions. The species used were: the manini, *Acanthurus sanvicensis*, a butterfly fish, *Chaetodon miliaris*, a filefish, *Pervagor spilosoma* and the puffer, *Arothron hispidus*. The mucous secretions of the first 3 species were non-toxic to mice but the secretions of the puffer, *Arothron hispidus*, were more toxic to mice than boxfish.
secretions. *Arothron hispidus* is known to contain a highly toxic substance, tetraodotoxin, in its flesh and viscera. The toxic mucous secretions of this species are further discussed in Chapter 12.
CHAPTER 6

ESTABLISHMENT OF A
STANDARD BIOASSAY FOR OSTRACIN

To detect ostracin in the mucous secretions of individual boxfish and to measure the potencies of various ostracin preparations, it was necessary to develop a standard bioassay which would not only be sensitive and reliable, but simple to perform. A bioassay using juvenile fish met these specifications.

CHOICE OF ASSAY FISH

The choice of a suitable bioassay fish depended upon its availability, hardiness, size and sensitivity to ostracin. A number of reef fishes were screened and, although all were very sensitive to the toxin, they were found lacking in other qualifications such as availability throughout the year and general hardiness. The most suitable test species was a brackish water cyprinodont, the sailfin molly, Molliesia latipinna which had been introduced into some of the streams and brackish water ponds on Oahu for mosquito control, and are therefore known, along with a few other cyprinodont fishes as "mosquito fish" by local residents.

The sailfin molly was very abundant in the brackish ponds and canals in Ala Moana park in Honolulu and thousands of these fish could be collected by seining the canal in
a single afternoon. The seasonal fluctuation in their numbers was only slight and these fish could be collected throughout the year. This was not true of most reef fishes whose numbers, especially of the young, fluctuated widely during a single year.

The sailfin molly met all the requirements of a good assay species. Besides being readily available, this euryhaline fish was quite hardy and could survive as well in fresh water as in sea water. Since all mollies are livebearers, their young are precocious and very hardy in comparison to the fry of oviparous fish. The newly born molly was about 8 - 10 mm in total length and could be reared easily in an aquarium. Because of the small size and availability of newborn mollies, it was decided to use only these and juvenile mollies for the bioassay. The use of such a small fish for a bioassay had obvious advantages. A small volume of water and consequently, a small amount of toxin could be used for testing.

Since it was not always possible to collect newborn mollies in the canal, as they are quickly eaten by predators, pregnant mollies were collected and kept in 35 - 45 gallon aquaria partitioned by hardware cloth. The newly born mollies would swim through the wire screen after being chased by the adults and take shelter at the safe end of the aquarium. They could be collected with a dipnet and
transferred to another aquarium. Hundreds of young mollies would be produced in a tank containing about 3 dozen pregnant females. The spent females were removed from the breeding tank and released or disposed of, as they preyed upon the young, competed with the others for food or died and fouled the water. Either brackish or sea water was used in these rearing tanks as it did not appear to affect the survival rate of young or adults.

Another assay fish which was used for many experiments was the African mouth-breeding cichlid, *Tilapia mossambica*. The young tilapias (10 - 12 mm total length) could be collected in one of the brackish ponds in Ala Moana park during the summer months. This species was also found in fresh and brackish waters throughout the Hawaiian Islands and was as hardy and as adequate an assay species as the sailfin molly. However, the supply of these fish was too erratic to satisfy its use as a standard assay species.

**DOSE RESPONSE DATA**

**Crude Ostracin**

The mucous secretions of a juvenile boxfish (about 90 mm total length) were collected in a 50 ml distilled water rinse and assayed with sailfin mollies in 100 ml of sea water. The experiment was conducted at room temperatures (25 - 26°C) and 0.02 to 10.0 ml of the 50 ml boxfish rinse were added to beakers of 100 ml of sea water, each containing 4 assay fish. Complete mortalities occurred in all
concentrations tested but only the 50% mortality time was recorded and plotted. The resulting curve obtained by plotting dose against survival time is shown in Figure 6.

The assay curve obtained was very similar to the curves obtained by Abbott and Ballantine (1957) for Gymnodinium toxin and by Hashimoto and Yasumoto (1960) for starfish saponin. An abrupt change in the course of the curve was characteristic of all these curves.

**Semi-pure Ostracin**

An aqueous stock solution of 1 mg/ml semi-pure ostracin was added to sea water to prepare dilutions of ostracin of 1:25,000 (40 ppm) to 1:20,000,000 (0.05 ppm). Four sailfin mollies were used per beaker containing 50 ml of sea water and the mean survival time was recorded (obtained by averaging individual deaths).

Complete mortality occurred in dilutions as low as 1.5 ppm (1:67,000) and a 50% mortality was noted at 0.1 ppm (1:10,000,000). At 40 ppm the mean survival time was 8.0 minutes and at 1.5 ppm it was 2 hours and 43 minutes.

The dose response curve resembles the curve obtained with crude ostracin (see Figure 7).

**THE STANDARD DOSE**

To quantitatively determine the concentration of ostracin secreted by stressed boxfish, a standard procedure of collecting and assaying whole crude secretions was adopted.
Figure 6. Dose-response curve of grade ostracin on agefin molluscs in sea water (4 fish/100 ml sea water)

Figure 7. Dose-response curve of semi-pure ostracin on agefin molluscs in sea water (4 fish/50 ml sea water)
A general description of the collection of boxfish secretions has already been given. In standardizing the procedure, the boxfish were kept under stress for five minutes and the volume of the rinse water was recorded. From 10 to 50 ml of distilled or sea water was used depending upon the size of the boxfish.

The dilute secretions were then assayed by pipetting a measured amount into 100 ml of sea water containing from 2 to 6 assay fish. A standard dose (1/50 of rinse) was arbitrarily chosen that corresponded to the inflection point on the dose-response curve. Hence, 1.0 ml of a 50 ml rinse, 0.5 ml of a 25 ml rinse and 0.2 ml of a 10 ml rinse were designated as standard doses. The mean survival time (determined by averaging individual deaths) or the time of 50% mortality, was recorded in minutes. Thus, if the mean survival time of 6 mollies at the standard dose was 8 minutes, the toxicity of the secretions was expressed as: TSD = 8.0 (TSD: toxicity of the standard dose). This notation will be used for convenience throughout the thesis and unless otherwise stated will denote the mean survival time, in minutes, of sailfin mollies immersed in 100 ml of sea water to which one standard dose of crude diluted boxfish mucous secretions has been added. If less than 100 ml of sea water was used for the assay, a proportional reduction would be made in the standard dose.
Advantages and Disadvantages of Fish Bioassay

The advantages for using such a bioassay instead of the conventional mouse assay were many: (1) it was rapid and easy to use; it could be readily carried out in the field with fairly crude equipment. No animal colonies, syringes or extraction solvents were required. (2) Sensitivity was high; less than .05% of the total ostracin secreted by a single boxfish was needed for a response. (3) Variability of assay fish was minimal; at the standard dose the 50% mortality differed from the 100% mortality by usually only one or two minutes. At smaller doses this difference increased slightly (5 - 10 minutes). (4) Size of assay fish had little effect on survival rate; within the range used (10 - 30 mm total length) the size of the molly had little effect on mean survival time. (5) Comparisons of toxicity were possible; the amount of ostracin secreted by individual boxfish could be estimated and the relative toxicity of crude and semi-pure preparations of ostracin could be accurately determined.

The disadvantages of this standard bioassay were: (1) the standard dose was a little too large; a smaller dose would have been more precise, however this would have extended the survival time and introduced variables such as possible decomposition of ostracin; (2) it was assumed that the imposed stress conditions caused nearly maximal ostracin secretion although all boxfish may not have
responded alike to the imposed stress situation.

**Symbols and Definitions**

The following symbols will be used for convenience throughout the thesis:

\[ SD = \text{the standard dose; } 1.0 \text{ ml of a } 50 \text{ ml rinse or its equivalent added to } 100 \text{ ml of sea water or equivalent containing 2 or more sailfin mollies or other specified species.} \]

\[ TSD = \text{toxicity at the standard dose expressed in the mean or } 50\% \text{ survival rate of the assay fish in minutes.} \]

\[ T(l:...) = \text{the toxicity of a semi-pure ostracin solution in mean survival time, in minutes, of assay fish; the concentration of ostracin is given within the parenthesis.} \]

The standard bioassay = the fish immersion bioassay.
CHAPTER 7

FLUCTUATIONS IN OSTRACIN OCCURRENCE IN BOXFISH SECRETIONS: ECOLOGICAL CONSIDERATIONS

Many poisonous marine organisms show great fluctuations in toxicity. The ciguatera-type toxins found in certain coral reef fishes are known to be of regional occurrence and are believed to have their origin in a food chain (Banner et al., 1960). The occurrence of Tetraodotoxin in the flesh and viscera of several species of puffer, or balloon fishes, undergo wide fluctuations, and one investigator (Tani, 1945) has suggested a seasonal correlation, with these fish being most toxic during the breeding season. The occurrence of red tides depends upon an unknown complex of hydrographic factors and consequently the occurrence of paralytic shellfish poisons has been sporadic and unpredictable (Schantz, 1960).

Therefore, it seemed reasonable to determine whether great fluctuations also occur in the toxicity of boxfish secretions and to determine if ostracin is an exogenous or endogenous toxin.

MATERIALS AND METHODS

The mucous secretions of about 150 boxfish were individually collected and bioassayed during a period from July 1959 to June 1961. The standard fish bioassay for ostracin was applied to the secretions of about 110 boxfish.
The total length of each boxfish was recorded and the sex was determined, when possible, by secondary sexual characters. Toxicity values (TSD) were recorded for each fish and used as an estimate of relative toxicity. Comparisons of TSD values were made with respect to size, sex, time of year captured and diet of boxfish.

**GENERAL TOXICITY**

Of the mucous secretions of about 110 boxfish assayed for toxicity by the standard bioassay, 104 boxfish secretions were toxic while only 6 were non-toxic. However, the "non-toxic" secretions were actually found to be weakly toxic when the standard dose was increased. Thus, the frequency occurrence of ostracin as estimated by the fish immersion assay was virtually 100%.

The relative toxicity of individual boxfish secretions was analyzed by establishing arbitrary toxicity categories. The toxicity values (TSD) of 73 boxfish as estimated by the standard bioassay are summarized in the following table:

<table>
<thead>
<tr>
<th>Toxicity category</th>
<th>TSD value</th>
<th>no. of boxfish</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly toxic</td>
<td>less than 10</td>
<td>30</td>
<td>41.1</td>
</tr>
<tr>
<td>Moderately toxic</td>
<td>10 - 25</td>
<td>28</td>
<td>38.4</td>
</tr>
<tr>
<td>Weakly toxic</td>
<td>more than 25</td>
<td>9</td>
<td>12.3</td>
</tr>
<tr>
<td>&quot;Non-toxic&quot;</td>
<td></td>
<td>6</td>
<td>8.2</td>
</tr>
</tbody>
</table>
Although this sample of 73 boxfish represents an assortment of various-sized fish caught under different conditions at different times of the year, almost 92% of the total had secretions sufficiently toxic to be readily detected by the standard bioassay. Since the boxfish secretes ostracin only under stress, the actual variations in toxicity may even be smaller than shown here because it is almost impossible to precisely control the stress involved in the capture of these fish. Some ostracin is probably lost during capture and handling.

SIZE AND TOXICITY

The smallest boxfish tested for toxicity (39 mm total length) by the standard assay had a TSD of 8.5 which puts it in the highly toxic category. The largest boxfish (178 mm total length assayed for ostracin had a TSD of 3.0. Higher toxicity values are expected in larger fish, however it is evident that the standard dose does not give a true picture of ostracin secretion in the larger boxfish. The significant point here is that even very young boxfish secrete a highly toxic "mucus" and there appears to be no deficiency in ostracin secretion in juvenile boxfish. The average TSD values of 3 size groups arranged arbitrarily are given below:

<table>
<thead>
<tr>
<th>No. of boxfish</th>
<th>Total length in mm</th>
<th>Mean TSD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>39 to 79</td>
<td>14.0</td>
<td>7.0 to 34.0</td>
</tr>
<tr>
<td>24</td>
<td>80 to 100</td>
<td>12.8</td>
<td>7.0 to 28.5</td>
</tr>
<tr>
<td>12</td>
<td>101 to 178</td>
<td>11.5</td>
<td>3.0 to 26.0</td>
</tr>
</tbody>
</table>
These values indicate that the smaller boxfish may secrete more ostracin in proportion to their size than the larger individuals, suggesting that the toxic secretions may have greater survival value to the smaller boxfish because of the larger number of predators that these fish are exposed to.

SEASONAL EFFECTS ON TOXICITY

Climatic fluctuations are slight in tropical oceanic waters. Although annual mean ocean surface temperatures around the Hawaiian Islands vary less than 10°C, seasonal fluctuations do occur in the inshore fauna and flora. Nevertheless, the secretions of boxfish captured at different times of the year showed no significant differences in toxicity during a two year period as demonstrated by TSD values of groups of boxfish collected during the winter, spring and summer of 1960 given below:

<table>
<thead>
<tr>
<th>No. of boxfish</th>
<th>Season</th>
<th>Mean TSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>Winter (December to March)</td>
<td>16.6</td>
</tr>
<tr>
<td>23</td>
<td>Spring (April to June)</td>
<td>13.3</td>
</tr>
<tr>
<td>18</td>
<td>Summer (July to October)</td>
<td>11.0</td>
</tr>
</tbody>
</table>

The slight differences in mean TSD values may be caused by the gradual increase in the assay water temperature since all bioassays were conducted at room temperatures.
EFFECT OF LOCALITY ON TOXICITY

Most boxfish investigated were captured in Kewalo Basin-Ala Moana reef area in Honolulu. The few boxfish captured at Diamond Head beach park, Kaneohe Bay, Punaluu and Nanakuli, Oahu were as toxic as Kewalo-Ala Moana fish.

EFFECT OF AGE AND SEX ON TOXICITY

Juvenile boxfish, as well as mature males and females, secrete ostracin under stress. No obvious differences in secretory toxicity was found among male and female fish although juvenile boxfish appeared to be almost as toxic as adults.

EFFECT OF METHOD OF CAPTURE ON TOXICITY

While the secretions of net-captured boxfish were usually highly toxic, those of boxfish caught in fish traps were often less so. The average TSD values for trap-caught fish were about 17.0 to 18.0 while for net-captured fish they were about 11.0 to 12.0.

Since it is known that ostracin is only secreted under stress, the prolonged stress conditions caused by confinement in a fish trap might cause a premature loss of ostracin.

TOXICITY OF BOXFISH IN CAPTIVITY

Boxfish collected in Kewalo Basin during February - July 1960 were routinely assayed for toxicity and held
in captivity for varying periods of time. These captive boxfish were then periodically assayed for ostracin secretions to determine whether a loss in toxicity occurred during confinement.

One group of fish was kept in a 100-gallon aquarium and fed frozen brine shrimp and a commercial trout food (Purina fry food). However, boxfish, especially the larger ones, were often reluctant to feed in captivity. Thus, only 4 boxfish were observed feeding regularly (feeding group) while 7 boxfish would not eat the prepared diet (non-feeding group).

Another group of boxfish was kept in a wire live cage in Kewalo Basin and was not fed (starved group).

The TSD values of secretions collected from these 3 groups are presented in Table II. These data show a great variability in ostracin secretion of captive boxfish, but firm conclusions cannot be made because too many variables were uncontrolled and too few boxfish would feed in captivity.

Nevertheless, it was clear that the capacity of boxfish of all 3 groups to secrete ostracin under stress was not greatly impaired by confinement of 2-3 weeks. This period was extended much longer in feeding fish. In one instance a moderately high level of ostracin was found in a feeding boxfish after almost 3 months in an aquarium while the ostracin secretion of the other 3 feeding fish
TABLE II. OSTRACIN SECRETION BY CAPTIVE BOXFISH AS ESTIMATED BY THE STANDARD BIOASSAY

<table>
<thead>
<tr>
<th>Total length (mm)</th>
<th>Initial TSD</th>
<th>Days in captivity</th>
<th>Subsequent TSD(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEEDING FISH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>53(^2)</td>
<td>19.0</td>
<td>17</td>
<td>16.0</td>
</tr>
<tr>
<td>64</td>
<td>15.0</td>
<td>32</td>
<td>9.5</td>
</tr>
<tr>
<td>68</td>
<td>25.0</td>
<td>53</td>
<td>111.0</td>
</tr>
<tr>
<td>75</td>
<td>...</td>
<td>3</td>
<td>34.0</td>
</tr>
<tr>
<td></td>
<td>NON-FEEDING FISH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>14.5</td>
<td>16</td>
<td>15.0</td>
</tr>
<tr>
<td>81</td>
<td>8.0</td>
<td>22</td>
<td>20.0</td>
</tr>
<tr>
<td>89</td>
<td>18.0</td>
<td>10</td>
<td>24.5</td>
</tr>
<tr>
<td>90</td>
<td>8.0</td>
<td>16</td>
<td>41.0</td>
</tr>
<tr>
<td>95</td>
<td>7.0</td>
<td>5</td>
<td>non-toxic at SD(^2)</td>
</tr>
<tr>
<td>95</td>
<td>20.0</td>
<td>5</td>
<td>non-toxic at SD</td>
</tr>
<tr>
<td>122</td>
<td>9.0</td>
<td>22</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>STARVED FISH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>...</td>
<td>20</td>
<td>22.0</td>
</tr>
<tr>
<td>93</td>
<td>...</td>
<td>3</td>
<td>11.0</td>
</tr>
<tr>
<td>119</td>
<td>...</td>
<td>10</td>
<td>8.0</td>
</tr>
<tr>
<td>120</td>
<td>...</td>
<td>13</td>
<td>non-toxic at SD</td>
</tr>
<tr>
<td>125</td>
<td>...</td>
<td>12</td>
<td>9.5</td>
</tr>
<tr>
<td>132</td>
<td>...</td>
<td>12</td>
<td>9.0</td>
</tr>
<tr>
<td>138</td>
<td>...</td>
<td>10</td>
<td>16.0</td>
</tr>
</tbody>
</table>

\(^1\)TSD: toxicity at standard dose.
\(^2\)This boxfish had no tail.
\(^3\)SD: standard dose.
first sharply increased in captivity, then gradually became reduced.

The non-feeding and starved boxfish became emaciated (marked by a "caving in" of the sides of the carapace) and rarely lived for more than a month in captivity. Unfortunately these (starved) fish were not assayed after 3 weeks because a tsunami destroyed the live cage in which they were being kept and all fish were lost. Nevertheless, some of these boxfish contained a moderately high amount of ostracin in their mucous secretions when assayed 10 to 20 days after capture.

**DISCUSSION**

The investigation of variabilities in the occurrence of ostracin in boxfish mucous secretions was prompted by the great fluctuations of other marine biotoxins of both exogenous and endogenous origin. The slight fluctuations found in ostracin secretion of freshly captured fish appeared to be only those normally expected due to experimental error and did not suggest environmental influences.

The consistency of ostracin occurrence in secretions of various-sized boxfish captured in different places at different times of the year, and the capacity of aquarium boxfish, maintained on an unnatural diet, to secrete ostracin under stress indicate that ostracin is metabolically produced by the boxfish independent of specific
precursors of environmental origin. Thus, oestracin is, in all probability, an endogenous toxin.
CHAPTER 8

GENERAL PHYSICAL AND CHEMICAL PROPERTIES OF OSTRACIN

The refinement of a preparative isolation procedure for ostracin depended upon the physical and chemical characteristics of the toxin. Knowledge of such properties as solubility and stability of ostracin were of primary importance in deciding upon an appropriate separation system. Both crude and semi-pure ostracin solutions were used in determining the general nature of the toxin.

STABILITY OF AQUEOUS OSTRACIN SOLUTIONS

The stability of crude ostracin solutions, either when frozen or at room temperatures, was very unpredictable. Initially, the crude secretions were collected and stored frozen in sea water, however a great reduction in toxicity was often noted after frozen samples were thawed and bioassayed. Toxicity data on crude sea water ostracin solutions stored frozen and at room temperatures have been presented in Table III. The only significance of these data was its great variability.

Some breakdown of active compounds was expected at room temperatures but the significant loss in toxicity that occurred in some frozen samples was surprising. This unexplained instability of crude ostracin in sea water
### TABLE III. THE STABILITY OF CRUDE OSTRACIN SOLUTIONS STORED FROZEN AND AT ROOM TEMPERATURES

<table>
<thead>
<tr>
<th>Volume of solution (ml)</th>
<th>Initial TSD</th>
<th>Duration of storage</th>
<th>Final TSD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FROZEN IN SEA WATER</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>7.0</td>
<td>62 days</td>
<td>6.5</td>
</tr>
<tr>
<td>10</td>
<td>9.0</td>
<td>2 days</td>
<td>12.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>85 days</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.5 (5xSD)</td>
</tr>
<tr>
<td>40</td>
<td>16.5</td>
<td>41 days</td>
<td>52.0 (5xSD)</td>
</tr>
<tr>
<td>50</td>
<td>7.0</td>
<td>1 day</td>
<td>10.0</td>
</tr>
<tr>
<td>50</td>
<td>9.0</td>
<td>1 day</td>
<td>43.0</td>
</tr>
<tr>
<td>50</td>
<td>6.0</td>
<td>51 days</td>
<td>34.0</td>
</tr>
<tr>
<td>50</td>
<td>7.0</td>
<td>36 days</td>
<td>9.0</td>
</tr>
<tr>
<td><strong>STORED IN SEA WATER AT ROOM TEMPERATURES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>7.0</td>
<td>1 day</td>
<td>10.0</td>
</tr>
<tr>
<td>50</td>
<td>9.0</td>
<td>1 day</td>
<td>non-toxic</td>
</tr>
<tr>
<td>50</td>
<td>7.0</td>
<td>4 days</td>
<td>non-toxic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63.0 (3xSD)</td>
</tr>
<tr>
<td>50</td>
<td>6.0</td>
<td>3 days</td>
<td>52.0 (5xSD)</td>
</tr>
<tr>
<td>100</td>
<td>8.0</td>
<td>1½ hours</td>
<td>4.0</td>
</tr>
<tr>
<td>500</td>
<td>5.0</td>
<td>3 days</td>
<td>11.0</td>
</tr>
</tbody>
</table>
led to future collection and storage of boxfish secretions in distilled water. Toxicity was better retained in frozen distilled water solutions, although in one notable instance when the combined mucous secretions of about 25 trap-caught boxfish were frozen in distilled water, they were only very weakly toxic when thawed and assayed 1 month later.

Thus, the unpredictable reduction in the activity of crude ostracin solutions prohibited prolonged storage of crude secretions and necessitated early treatment of samples.

HEAT STABILITY OF OSTRACIN

Ostracin was found to be a very thermostable toxin. Boiling crude ostracin solutions had no effect on toxicity. Samples of ostracin in sea water and distilled water were heated for a total of 3 hours in a hot water bath (91 - 94°C) and aliquots were assayed for toxicity at intervals of 15, 40, 105 and 180 minutes.

<table>
<thead>
<tr>
<th>Ostracin solution</th>
<th>Initial TSD</th>
<th>Final TSD (after 3 hrs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water</td>
<td>8.0</td>
<td>8.5</td>
</tr>
<tr>
<td>sea water</td>
<td>11.0</td>
<td>23.0</td>
</tr>
</tbody>
</table>

The reduction in the toxicity of the sea water - ostracin solution occurred during the first 15 minutes and no further

* TSD's recorded at intervals destroyed by fire; only final TSD value available.
change was noted, again emphasizing the greater instability of ostracin in sea water (see preceding table).

It was possible to boil an aqueous ostracin solution to dryness without appreciable loss in toxicity. In fact, boiling an ostracin solution for a few minutes appeared to have a stabilizing effect on toxicity as such solutions could be stored for prolonged periods at room temperature or under refrigeration without serious loss in toxicity.

ACID-ALKALINE STABILITY OF OSTRACIN

Ostracin was relatively stable over a pH range from 2.0 to 11.6, however with the addition of excess strong base (KOH) ostracin was completely detoxified.

To determine the effect of pH on ostracin, a 0.1% ostracin solution in 0.9% NaCl was prepared and 1 ml of this solution was pipetted into several vials to which was added 1 ml of an appropriate phosphate buffer to obtain a pH range of about 4.0 to 9.0. Acetic acid and 1.0% KOH were added to other vials to extend the pH range beyond 4.0 and 9.0. The pH was recorded with a Beckman Zeromatic pH meter and the vials were refrigerated for 36 hours. The pH was then recorded again and an aliquot from each vial was assayed for toxicity by the standard bioassay.

A second experiment was performed using distilled water ostracin solutions made alkaline by addition of 1.0% KOH and refrigerated for 45 hours. The data of both experiments
are presented in Table IV.

The data of these experiments indicate that the effect of acidity or alkalinity was not a significant factor in the detoxification of ostracin samples.

POSSIBLE BACTERIAL DECOMPOSITION OF OSTRACIN

The instability of crude ostracin solutions and the stability of heated ostracin solutions suggested a possibility of bacterial detoxification. Experiments were performed to ascertain the possible role of bacteria in detoxification. Samples of semi-pure ostracin and crude ostracin in distilled water were treated as follows: control (no treatment); heated to boiling and aseptically stoppered; heated to boiling and allowed to cool before stoppering (not aseptic); addition of an antibiotic (streptomycin sulfate); filtration through a bacterial filter (pore size 0.45 μ). All solutions were kept in test tubes at room temperatures and tested after an interval of 51 days and some after 72 days. The results are shown in Table V.

The only significant losses in toxicity occurred in the crude ostracin solutions except when these solutions were heated. Apparently the antibiotic and bacterial filter were less effective than heating, indicating that perhaps conditions were not really aseptic.

To determine whether crude boxfish secretions contained
TABLE IV. THE EFFECT OF pH ON
THE STABILITY OF OSTRACIN

<table>
<thead>
<tr>
<th>1st experiment</th>
<th>Initial pH</th>
<th>pH after 36 hrs.</th>
<th>T(1:100,000)&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.8</td>
<td>2.0</td>
<td>35.5</td>
</tr>
<tr>
<td></td>
<td>2.6</td>
<td>2.6</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>4.8</td>
<td>29.0</td>
</tr>
<tr>
<td></td>
<td>5.1</td>
<td>5.2</td>
<td>29.0</td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>5.9</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>6.5</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>7.4</td>
<td>non-toxic</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>11.5</td>
<td>12.2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean survival time in minutes of two sailfin mollies in 25 ml of sea water containing 0.25 mg of ostracin (10 ppm).

<table>
<thead>
<tr>
<th>2nd experiment</th>
<th>Initial pH</th>
<th>pH after 45 hrs.</th>
<th>T(1:50,000)&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>6.3</td>
<td>6.4</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>8.1</td>
<td>7.2</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.0</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>9.7</td>
<td>10.2</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>10.9</td>
<td>10.9</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>11.6</td>
<td>26.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean survival time in minutes of two sailfin mollies in 50 ml of sea water containing 1.0 mg of ostracin (20 ppm).
TABLE V. COMPARISON OF THE DETOXIFICATION RATE OF SEMI-PURE AND CRUDE OSTRACIN—DISTILLED WATER SOLUTIONS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Toxicity to assay fish&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Before</th>
<th>51 days later</th>
<th>72 days later</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEMI-PURE OSTRACIN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.0</td>
<td>14.0</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>Antibiotic</td>
<td>. . .</td>
<td>15.0</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>Heated and sealed at once</td>
<td>. . .</td>
<td>14.5</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>Heated and sealed after cooling</td>
<td>. . .</td>
<td>15.0</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>Filtered (bacterial)</td>
<td>. . .</td>
<td>15.5</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>CRUDE OSTRACIN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.0</td>
<td>29.0</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>Antibiotic</td>
<td>. . .</td>
<td>70.0</td>
<td>199.5</td>
<td></td>
</tr>
<tr>
<td>Heated and sealed at once</td>
<td>. . .</td>
<td>13.0</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Heated and sealed after cooling</td>
<td>. . .</td>
<td>19.0</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>Filtered (bacterial)</td>
<td>. . .</td>
<td>32.0</td>
<td>non-toxic</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean survival time in minutes of four sailfin mollies in 50 ml of sea water containing an equal unit of ostracin.
a detoxifying agent (such as an enzyme) or if sea water itself could cause detoxification, another experiment was performed using crude and semi-pure ostracin in sea water solutions kept at room temperatures. The results are shown in Table VI.

Since heated sea water-ostracin solutions retained toxicity and unheated ostracin-sea water solutions lost their toxicity to a considerable extent, it appeared that sea water in itself was not responsible for detoxification although the possibility still exists that a detoxifying agent (enzyme) occurs in the crude secretions.

Possible detoxification by oxidation could be another explanation. Since heating would drive off most of the dissolved oxygen from an ostracin solution, its stability could be explained by lack of oxygen. Preliminary experiments showed that bubbling pure oxygen or air through an ostracin-sea water solution for 1 hour caused considerable loss in toxicity. On the other hand, bubbling oxygen through a heated ostracin-sea water solution for 1 hour caused no appreciable loss in toxicity, indicating that oxygen per se does not cause detoxification.

In conclusion, it appears that bacterial action is at least partly responsible for losses in toxicity of ostracin samples. Abbott and Ballantine (1957) noted that the toxicity of Gymnodinium cultures decreases when the bacterial population becomes high, although Shilo and
TABLE VI. DETOXIFICATION OF CRUDE AND PURE OSTRACIN IN SEA WATER SOLUTIONS

<table>
<thead>
<tr>
<th>Substance</th>
<th>Toxicity to assay fish(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Semi-pure ostracin (1:20,000)</td>
<td>14.5</td>
</tr>
<tr>
<td>Crude ostracin control no. 1</td>
<td>7.0</td>
</tr>
<tr>
<td>Crude ostracin control no. 2</td>
<td>. . .</td>
</tr>
<tr>
<td>Crude ostracin heated no. 1</td>
<td>10.0</td>
</tr>
<tr>
<td>Crude ostracin heated no. 2</td>
<td>. . .</td>
</tr>
</tbody>
</table>

\(^1\)Mean survival time in minutes of four sailfin mollies in 50 ml of sea water containing an equal unit of ostracin.
Rosenberger (1960) reported that the *P. parvum* toxins undergo rapid inactivation under field conditions, and even in bacteria-free laboratory cultures. The loss in toxicity of frozen ostracin samples suggest that other factors, such as detoxifying enzymes, are operating since rapid inactivation occurred only in crude, unheated secretions.

**SOLUBILITY OF OSTRACIN**

Solubilities were determined by heating an ostracin solution to dryness, weighing the residue and mixing it in the desired solvent at room temperature. The solution was then centrifuged and the supernatant fluid was filtered. The filtrate was dried and the residue dissolved into water and assayed for toxicity.

Ostracin was very soluble in water and was less soluble in other polar solvents such as methanol, ethanol and acetone. The solubility of ostracin was greater in absolute methanol and 50 to 95% ethanol than in absolute ethanol and ostracin was only partly soluble in acetone. Ostracin was insoluble in the non-polar solvents diethyl ether and benzene, but was very soluble in chloroform. Liquid-liquid extraction of ostracin from distilled water by chloroform was successful, although after vigorous shaking of these liquids in a separatory flask and standing over-night, the water layer was significantly more toxic than the chloroform layer (*T* 1:50,000 = 10.0 of the water)*

* Since ostracin is a relatively large molecule it forms colloidal dispersions rather than true solutions.
layer and 18.5 of the chloroform layer. None of the specified solvents caused detoxification.

DIALYSIS OF OSTRACIN

Ostracin, as mentioned previously, was non-dialyzable, indicating that it is a rather large molecule in colloidal solution. Ostracin shares this property with many other marine ichthyotoxins, including the sea cucumber toxin, holothurin A, the red tide toxins of Gymnodinium veneficum and Prymnesium parvum and the starfish poisons.

FOAMING OF OSTRACIN SOLUTIONS

Stable foams are very characteristic of ostracin solutions and there appeared to be a direct correlation of toxicity with degree of foaminess. The most toxic solutions foamed readily and profusely when agitated and the general toxicity of an ostracin solution could be estimated by its capacity to produce a stable, rich foam when shaken.
CHAPTER 9

SPECIAL CHARACTERISTICS OF OSTRACIN

The superficial resemblance of ostracin to holothurin A, the steroid saponin from the sea cucumber Actinopyga agassizi (Nigrelli and Jakowska, 1960), the starfish saponin (Hashimoto and Yasumoto, 1960) and the red tide toxins from Gymnodinium veneficium (Abbott and Ballantine, 1957) and Prymnesium parvum (Shilo and Rosenberger, 1960) suggested that ostracin might be a saponin-like compound.

Saponins* in general have diagnostic properties which distinguish them from other toxins. They are poisonous to fish and many other cold-blooded animals; they are surface-active agents which produce a soapy lather in aqueous solutions; and they are hemolytic, i.e., they lyse red blood cells. Furthermore, steroid saponins, such as holothurin and digitonin, form stable, non-toxic complexes with cholesterol.

Since ostracin had been shown to be ichthyotoxic and to form soap-like foams, its effect on erythrocytes and its reaction with cholesterol was studied.

---

* The general term "saponin" refers to all the plant glycosides which possess these properties (e.g., digitonin). Unfortunately, this term also refers to a specific compound with an empirical formula of \( C_{12}H_{52}O_{17} \). To avoid confusion, this saponin will be referred to as saponin (Merck).
EFFECT OF OSTRACIN ON RED BLOOD CELLS

Whole blood was collected from the hearts of several species of bony fishes and stored in a sodium citrate solution (8 g sodium citrate, 7.25 g sodium chloride and 20.5 g dextrose per liter distilled water) specially prepared by the serological laboratory of the Honolulu Biological Laboratory (U.S. Bureau of Commercial Fisheries) for tuna blood. The red blood cells (RBC) were washed several times with citrate-saline and stored under refrigeration. Human, mouse, rabbit and toad erythrocytes were prepared and stored in a similar manner.

Qualitative Test for Hemolysis and Agglutination

Semi-pure ostracin dissolved in citrate-saline was drawn into a capillary tube (diameter 1.3 - 1.5 mm; length 75 mm). The wet end of this capillary tube was immersed in a washed RBC suspension to draw the erythrocytes into the tube. The dry end was sealed with modeling clay and the capillary tube was set vertically in a rack. After the RBC passed through the ostracin solution and settled at the bottom of the tube, a reading was taken by comparing the appearance of the RBC suspension with a saline control. If hemolysis occurred, hemoglobin from the lysed RBC would color the suspension a uniform red. If agglutination occurred, the RBC would clump as they settled in the tube.

Ostracin, at a dilution of 1:1,000 caused complete hemolysis of all RBC suspensions tested. At this concentration, strong agglutination of RBC preceded hemolysis.
in all fish and rabbit RBC, whereas no agglutination was observed in human or mouse RBC suspensions. Control solutions of crude holothurin (aqueous extract of cuvierian tubules of *Actinopyga obesa*) and saponin (Merck) caused complete hemolysis but no agglutination of all RBC used.

Agglutination of skipjack tuna blood occurred at ostracin dilutions as high as 1:20,000 whereas hemolysis was observed (in the capillary tubes) in dilutions as great as 1:100,000. The list of species used and their RBC reactions is given in Table VII.

The so-called agglutination reaction which occurred when ostracin was added to fish or rabbit erythrocytes superficially resembled an antibody-antigen agglutination but since this reaction could not be altered or destroyed by denaturing the protein impurities in the ostracin sample, it must be attributed to ostracin itself. Microscopical examination showed that a characteristic apparent reduction in size of the agglutinated and hemolyzed erythrocytes occurred within seconds after introduction of ostracin (see Figures 8 and 9).

Hemolysis and agglutination of boxfish erythrocytes by ostracin occurred in vitro and both reactions appeared as strong as those using other RBC. Undoubtedly some mechanism has evolved in the boxfish to protect the fish from its own toxin although complete protection from the active toxin has not been achieved judging by its harmful
TABLE VII. OSTRACIN HEMOLYSIS AND AGGLUTINATION OF THE ERYTHROCYTES OF SOME VERTEBRATES

<table>
<thead>
<tr>
<th>Species</th>
<th>Hemolysis</th>
<th>Agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conger marginatus (Conger eel)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gymnothorax sp. (Moray eel)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Katsuwonus pelamis (Skipjack tuna)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thunnus alalunga (albacore)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neothunnus macropterus (Yellowfin tuna)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Parathunnus sibi (Bigeye tuna)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Coryphaena hippurus (dolphin)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Abudefeduf abdominalis (mao mao)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acanthurus sanvicensis (manini)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scarus sp. (parrot fish)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ostracion lentiginosus (boxfish)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bufo marinus (toad)</td>
<td>+</td>
<td>..</td>
</tr>
<tr>
<td>Mouse</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Man</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+: reaction occurred
-: no reaction
ostracin concentration: 1:1,000
Figure 8. Erythrocytes of skipjack tuna (Katsuwomis pelamis) before addition of ostracin. x440.

Figure 9. The same erythrocytes seconds after addition of ostracin showing agglutination and hemolysis. Only the cell nuclei of the erythrocyte ghosts are visible. x440.
effect on erythrocytes and general toxicity to the boxfish (see pages 42 - 43).

The toxic action of ostracin on erythrocytes is probably not a direct cause of its lethal effect to assay fish since examination of the blood of fish killed by ostracin failed to show any evidence of hemolysis or agglutination.

**Quantitative Hemolytic Test for Ostracin**

To determine the relative potency of ostracin as a hemolysin, dilutions of semi-pure ostracin of 0.1 to 20 ppm were prepared to which 0.1 ml of a tuna RBC suspension was added. The test tubes were shaken and allowed to stand at room temperature for 1 - 2 hours. They were then centrifuged for 5 minutes and the supernatant fluid was transferred to clean test tubes to determine the percent transmittance at wavelengths of 510, 525 and 540 μm, using a Beckman Spectrocolorimeter. The transmittance of the RBC-saline control was set at 100%. The results of one experiment are graphed in Figure 10.

Some hemolysis of tuna blood occurred at very low ostracin concentrations (0.1 ppm). This roughly corresponds to the approximate LD50 of assay fish obtained with semi-pure ostracin (see Figure 7). The sensitivity of RBC to lysis by ostracin made a quantitative hemolytic bioassay possible. However, since such a bioassay required frequent time consuming standardization, it was deferred in favor of the simpler fish bioassay.
Figure 10. Hemolysis of skipjack tuna erythrocytes by ostracin measured with a spectrophotometer.
PRECIPITATION OF OSTRACIN BY CHOLESTEROL

At present, all saponins and sapogenins (the hydrolytic product of saponins) are divided into two large classes: the steroids and the triterpenoids. Although both types of saponins are hemolytic, only some steroid saponins form water-insoluble binary complexes with cholesterol (Ransom, 1901; Sokolskaya, 1951; Wall et al., 1952).

Holothurin A, the steroid saponin of the sea cucumber, forms a complex with cholesterol and this property has been useful in the purification of this toxin (Chanley et al., 1959). Abbott and Ballantine (1957) found that 20 mg of cholesterol added to 100 ml of sea water containing a highly toxic Gymnodinium culture gave complete protection to the assay fish. Although the authors did not give reasons for this, B.C. Abbott (personal communication) suspected that the Gymnodinium toxin was a saponin.

Thus, the saponin-like activity and properties of ostracin led to investigation of the reaction of ostracin with cholesterol.

Materials and Methods

First Experiment

Cholesterol was dissolved in hot 95% ethanol. Test tubes containing 4 mg of semi-pure ostracin in 95% ethanol were prepared and 20, 50 and 100 mg cholesterol, respectively, were added to 3 test tubes. Ethanol controls of both cholesterol and ostracin were also prepared and all solutions
were kept at room temperature. After 2 days, aliquots of each test tube were dried and the residues were assayed for toxicity by the fish standard assay and for hemolytic activity by the capillary tube method.

Second Experiment

The first experiment was repeated using 5 mg of a different ostracin preparation together with 10, 25 and 50 mg of cholesterol in 95% ethanol. The test tubes were refrigerated for 5 days and then assayed for toxicity as described in Experiment 1.

Third Experiment

About 20 mg of a semi-pure ostracin preparation (chloroform extract) was dissolved in absolute methanol containing 20 mg of cholesterol and refrigerated for 2 days. The residue was assayed for ichthyotoxic and hemolytic activity.

Results

Both the precipitate and the supernatant fluid of all ethanol cholesterol-ostracin solutions were non-toxic to assay fish, whereas the ethanol-ostracin control lost no toxicity. Besides losing its ichthyotoxic activity, cholesterol-treated ostracin was non-hemolytic and non-agglutinative when tested with fish RBC. The methanol ostracin-cholesterol solution retained some ichthyotoxicity and hemolytic-agglutinative activity, but heating an aqueous solution of the methanol cholesterol-ostracin residue for
a few minutes caused considerable reduction in total activity.

Some of the activity of the ostracin-cholesterol residue was restored after extraction with cold ether. Dissolving the ostracin-cholesterol complex in hot pyridine followed by extraction with cold ether of the residue after the pyridine had been removed by evaporation caused greater restoration of activity. Removal of the ether-soluble cholesterol left an ether-insoluble, water-soluble icathyo-toxic precipitate. No toxic precipitate was formed when ether was added to a pyridine solution of the ostracin-cholesterol complex.

**Discussion**

The relationship between hemolysis of red blood cells by saponins and precipitation of steroid saponins by cholesterol becomes clearer when we consider how a lytic substance acts. Danielli (1958) states that a lytic substance, such as saponin, penetrates into the cholesterol film surrounding the RBC to form a stable complex with the cholesterol. As saponin penetrates, it disperses the protein monolayer causing the leakage of hemoglobin, or hemolysis.

Ruyssen (1957) has shown that saponins, such as digitonin, have no solubilizing action on cholesterol as do other hemolytic agents, such as butanol, detergents, etc., and that an equimolar digitonin-cholesterol complex remained combined on the ghosts of the erythrocytes hemolyzed by digitonin. Thus, combining saponin with cholesterol prior
to addition to a RBC suspension prevents hemolysis.

Danielli (1958) compared the capacity of surface active or lytic agents to penetrate cholesterol monolayers and found that, in general, increase in number of -CH₂- groups in the molecule increased penetration and that the effectiveness of polar groups of such agents gave a series: 

\[ \text{NH}_3^+ > \text{SO}_4^- > \text{SO}_3^- > \text{COO}^- > \text{N(CH}_3)_3^+ \] . Danielli pointed out that whereas substances that penetrate and disperse protein monolayers are cytolytic, substances which are adsorbed on protein monolayers, but do not disperse them are agglutinative but not cytolytic. It was shown that ostracin, unlike holothurin and saponin (Merck), is both agglutinative and hemolytic. This suggests that initially ostracin is adsorbed on the protein layer of RBC causing agglutination, but as it eventually penetrates the cholesterol layer it causes dispersion of the protein monolayer and consequent hemolysis.

The parallelisms between ichthyotoxicity, hemolytic-agglutinative activity and precipitation by cholesterol strongly suggest that ostracin, the active substance in boxfish mucous secretions, is a steroid saponin.
As mentioned before, saponins have been defined as plant glycosides that have the distinctive property of forming a soapy lather in water (Fieser and Fieser, 1959). These glycosides are extremely effective as surface active agents because of their ability to lower surface tension. This property is associated with their ability to alter the permeability of cell membranes and to hemolyze erythrocytes. Thus, saponins exert a general toxicity on most tissues, however certain saponins possess potent physiological properties which cannot be explained by their general surface tension effects. Such saponins are sometimes referred to as "sapotoxins," and holothurin A, the steroid saponin of the sea cucumber, is a prime example.

The principal products of the hydrolytic cleavage of saponins are sapogenins (aglycone portion) and sugars (glycosidic portion). The aglycone structure of a steroid saponin consists of a reduced cyclopentenophenanthrene ring with a spiroketoacetal side chain with methyl groups.

Certain steroid glycosides which possess similar properties as saponins but exert a characteristic physiological effect on the heart are classified as cardiac glycosides, e.g., digitoxin, one of the cardiac-active glycosides isolated from the leaves of the purple foxglove, Digitalis purpurea.
attached to the C₁₀ and C₁₃ atoms and with a hydroxyl (OH) group attached to the C₃ atom. The glycosidic portion (sugars) attaches to the C₃ hydroxyl group through a glycosidic linkage, or bond. The water solubility and pharmacological activity of saponins are due to this glycosidic portion. The sapogenins are usually biologically inactive because of their low solubility in water, but are often more soluble than saponins in other solvents such as ether, benzene, etc. (Sokolskaya, 1951).

The properties of ostracin, as elucidated in the preceding chapters, clearly indicate that this toxin is a saponin. Furthermore, the precipitation and detoxification of ostracin by cholesterol suggest that ostracin is a steroid saponin. Table VIII presents a comparison of the properties of ostracin with two commonly known saponins, digitonin and saponin (Merck). From these data it appears that ostracin is remarkably similar to both saponin (Merck) and digitonin in general properties with the exception of solubilities, wherein ostracin seems to be much more soluble in a wider range of solvents than these saponins.

Ostracin, however, is considerably more toxic to fish than saponin (Merck). A concentration of pure saponin (Merck) of 1:5,000 (200 ppm) was required to kill sailfin mollies within 30 minutes whereas a semi-pure ostracin concentration of only 1:250,000 (4 ppm) was lethal to these assay fish in the same time. Evidently ostracin,
<table>
<thead>
<tr>
<th>PROPERTY</th>
<th>OSTRAVIN</th>
<th>SAPONIN (MERCK)</th>
<th>DIGITONIN&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>unknown</td>
<td>$C_{32}H_{52}O_{17}$</td>
<td>$C_{55}H_{90}O_{29}$</td>
</tr>
<tr>
<td>Appearance</td>
<td>white amorphous hygroscopic powder (impure)</td>
<td>white amorphous powder (pure)</td>
<td>white crystalline powder (pure)</td>
</tr>
<tr>
<td>Foaming ability in water</td>
<td>produces stable foams</td>
<td>produces stable foams</td>
<td>produces stable foams</td>
</tr>
<tr>
<td>Solubility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>v. sol.</td>
<td>sol.</td>
<td>sl. sol.</td>
</tr>
<tr>
<td>methanol</td>
<td>sol.</td>
<td>...</td>
<td>sol.</td>
</tr>
<tr>
<td>ethanol</td>
<td>sol.</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>acetone</td>
<td>sl. sol.</td>
<td>...</td>
<td>v. sl. sol.</td>
</tr>
<tr>
<td>ether</td>
<td>insol.</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>benzene</td>
<td>insol.</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>chloroform</td>
<td>v. sol.</td>
<td>...</td>
<td>v. sl. sol.</td>
</tr>
<tr>
<td>Analysis</td>
<td>non-dialyzable</td>
<td>non-dialyzable</td>
<td>non-dialyzable</td>
</tr>
<tr>
<td>Heat stability</td>
<td>thermostable</td>
<td>thermostable</td>
<td>thermostable</td>
</tr>
<tr>
<td>Acid-base stability</td>
<td>stable in acid</td>
<td>stable in acid</td>
<td>stable in acid</td>
</tr>
<tr>
<td></td>
<td>unstable in alkali</td>
<td>unstable in alkali</td>
<td>unstable in alkali</td>
</tr>
<tr>
<td>Toxicity to fish</td>
<td>ichthyotoxic</td>
<td>ichthyotoxic</td>
<td>ichthyotoxic</td>
</tr>
<tr>
<td>Effect on RBC</td>
<td>agglutinative-hemolytic</td>
<td>hemolytic</td>
<td>hemolytic</td>
</tr>
<tr>
<td>Reaction with cholesterol</td>
<td>forms water-insoluble complex</td>
<td>forms water-insoluble complex</td>
<td>forms water-insoluble complex</td>
</tr>
</tbody>
</table>


<sup>2</sup>Data apply only to room and refrigeration temperatures; most saponins are hydrolysed by prolonged treatment with mineral acids at high temperatures.
perhaps because of its solubility properties, is biologically more active than saponin (Merck) and should be classified as a "sapotoxin" along with holothurin and the starfish saponin (see Chapter 19).

The resemblance of ostracin to steroid saponins is sufficient evidence to tentatively name the ichthyotoxic hemolysin in boxfish secretions "ostracin". Following the nomenclature rules for saponins, the sapogenin should then be named "ostracigenin". This does not dismiss the possibility of other toxins occurring in boxfish mucous secretions, but until different toxic fractions can be demonstrated, ostracin will be assumed to be a single toxin.
CHAPTER 11

PURIFICATION TECHNIQUES FOR OSTRACIN

Although the objectives of this thesis did not include complete purification of ostracin, a sufficiently pure preparation was needed to determine the chemical characteristics of the toxin. Many purification techniques were attempted, including methods used by investigators in isolating other sapotoxins. A summary of these techniques and results is discussed.

PRECIPITATION WITH CHOLESTEROL

Chanley et al. (1959) successfully purified holothurin A by precipitating it with cholesterol, reactivating the toxin with pyridine, and precipitating the glycoside from pyridine by ether. This procedure was attempted with ostracin, however, this substance could not be readily precipitated from a pyridine solution by ether and the method was abandoned.

CHROMATOGRAPHY

Foam Chromatography

Foam chromatography was tried because aqueous solutions of ostracin, like all saponins, produced very stable foams. Preliminary trials using compressed air bubbling through crude ostracin solutions were partly successful but a reduction in toxicity occurred in both the foam and the
Paper Chromatography

Paper chromatography has been successfully used to separate saponins (Dutta, 1955). Although not a preparative method, paper chromatography can be useful in determining the number of components in a mixture. Ascending paper chromatograms were prepared with the following solvent systems: n-butanol, glacial acetic acid and water (4:1:5); ethyl acetate, glacial acetic acid and water (3:1:3); and pyridine ethyl acetate and water (2:1:2). The papers were developed with ninhydrin, a silver nitrate-ammonium hydroxide solution and a spray reagent developed by Lemieux and Bauer (1954) for use in the detection of saponins.

The best separation occurred in the pyridine-ethyl acetate-water (2:1:2) solvent system but ostracin was eluted from an unstained portion of the chromatogram. The ninhydrin-stained band was non-toxic, and the Lemieux and Bauer spray reagent stained only the non-toxic solvent front. Unless a spray reagent is found that will color the toxic band of the chromatogram, paper chromatography of ostracin will have only limited value.

EXTRACTION WITH ORGANIC SOLVENTS

Acetone

Acetone soxhlet extraction* for 24 hours of the crude

* Done by David Boylan, Chemistry Department, University of Hawaii.
residue obtained after boiling an aqueous ostracin solution to dryness was successful. The oily yellowish acetone residue dissolved in water killed assay fish within 45 minutes at a concentration of 1:500,000.

**Chloroform**

The residue of a boiled aqueous ostracin solution was extracted with chloroform at room temperature. The ostracin in the residue was readily dissolved in chloroform and the chloroform residue had a toxicity comparable to that of the acetone residue.

**CENTRIFUGATION AND DIALYSIS**

Initially, all semi-pure ostracin solutions were prepared by centrifuging them at 15,000 rpm, discarding the non-toxic precipitate, and dialyzing the supernantant fluid against running tap water or distilled water. The toxic residue from dialysis was stored in distilled water. It was later discovered that heating the aqueous ostracin solution stabilized the activity of ostracin and that storage in chloroform was preferable to water since some detoxification occurred, either by bacterial decomposition or hydrolysis, in aqueous solutions.

Thus, the scheme recommended for preliminary purification of ostracin is presented in Figure 1. The semi-pure ostracin obtained in step IV was, in most instances, the preparation used for all toxicity experiments. The addition of the chloroform extraction was a recent refinement.
FIGURE 11. PRELIMINARY PURIFICATION PROCEDURE FOR OSTRACIN

Crude aqueous boxfish secretions

Filtered

- Ppt. (discarded)

Filtrate (I)

Centrifuged at 15,000 rpm for 30 minutes to 1 hour

- Ppt. (discarded)

Supernatant (II)

Boiled for 15 minutes and filtered

- Ppt. (discarded)

Filtrate (III)

Dialyzed for 6 hours or more

- Dialyzate (discarded)

Residue in aqueous solution (IV)

Heated or evaporated to dryness and extracted with chloroform

Chloroform insoluble residue (discarded)

Chloroform soluble fraction (V)

Concentrated by heating or evaporation

Syrup

Wash with ether

- Ether soluble residue (discarded)

Ppt: semi-pure ostracin (VI)
PREPARATION OF OSTRACIGENIN

An aqueous ostracin solution (fraction IV), acidified by adding concentrated HCl (1 ml concentrated HCl to 5 ml of 6 mg/m ostracin solution), was heated in boiling water for 1 hour. After 15 minutes a precipitate began to form and after 1 hour an oily syrup collected at the surface and sides of the glass container. This syrup was washed several times with water and after drying in an oven was completely dissolved in benzene. The toxicity of the acidified supernatant was considerably reduced, indicating that acid-hydrolysis occurred and that the syrupsy fraction was ostracigenin.
CHAPTER 12
TOXIC SECRETIONS OF OTHER PLECTOGNATHS

Reports of ichthyotoxins produced by trunkfishes (Clark and Gohar, 1953; Brock, 1955; Straughan, 1959) are suggestive that ostracin or ostracin-like toxins might be found throughout the trunkfish family. There are many examples of similar toxins occurring in closely related organisms. To cite a few marine examples, holothurin-like toxins have been reported in many genera of sea cucumbers (Yamanouchi, 1955; Nigrelli and Jakowska, 1960) and there appear to be similarities in red tide toxins from Gymnodinium venificum (Abbott and Ballantine, 1957) and Gonyaulax monilata (Gates and Wilson, 1960). The pufferfishes, which include several families, may also contain similar or identical toxins. The important unanswered question is whether a single toxin persists throughout a family or group of related families or whether analogous but distinctly different toxins occur in different species. The answer to this question could have important significance in understanding the phylogenetic relationships between species.

TRUNKFISH

Besides the boxfish, another common Hawaiian trunkfish is the cowfish, Lactoria cornuta. This species was often
encountered in shallow waters over sand flats while collecting boxfish. It is a rather inconspicuous, slow swimming trunkfish and was easily captured by dipnetting.

The mucous secretions of a few cowfish were collected and assayed for toxicity, and of 19 cowfish (48 to 102 mm total length), toxic secretions were detected in only 11 fish. The cowfish mucous secretions foamed as well as those of the boxfish, but the cowfish toxin appeared to be much more labile than ostracin. This toxin instability probably accounted for the 8 non-toxic fish, as merely filtering or centrifuging the cowfish toxin seemed to cause considerable detoxification. Cowfish secretions also caused hemolysis and agglutination of fish RBC indicating that the active principle may also be a saponin-like toxin.

The secretions of two other species of trunkfish were collected and assayed for ichthyotoxicity and hemolytic activity. One juvenile *Lactoria diaphanus* was captured in a mid-water trawl by the Charles H. Gilbert, research vessel of the U.S. Bureau of Commercial Fisheries and kept in a shipboard aquarium. The secretions were collected from the weakened trunkfish immediately, as it was taken from the trawl, and again a few days later. The samples were frozen and tested on shore. Only the second sample

* Collected by Robert A. Morris
was ichthyotoxic, and hemolyzed and agglutinated fish erythrocytes.

Two specimens of a rare trunkfish, tentatively identified as *Rhyncostracion* sp., were collected off Pokai Bay, Oahu and kept in an aquarium. The secretions of both fish were collected and assayed and found to be ichthyotoxic, agglutinative and hemolytic.

Thus, all four Hawaiian trunkfishes examined contained an ichthyotoxic hemolysin in their mucous secretions. The similarities and differences of these toxins to ostracin were not determined.

PUFFERS

It was mentioned previously that the mucous secretions of the puffer, *A. hispidus*, were highly toxic to mice. The puffer mucus was collected by placing a puffer in a beaker and rinsing the fish with distilled water. This aqueous mucous solution, injected intraperitoneally into white mice and fish (*Acanthurus saniticensis*), caused death within a few minutes, depending upon the dose.

The puffer mucous toxin, unlike ostracin, was non-hemolytic and non-ichthyotoxic, i.e., it was non-toxic to fish when dissolved in their aquarium water. However, application of this puffer toxin in Ringer's solution to an isolated sciatic nerve of the toad blocked the action

* Collected by Donald E. Morris and Robert A. Morris.
potentials of the nerve in less than 1 minute, indicating that a potent neurotoxin was present.

Two other species, the spiny puffer, *Diodon hystrix* and a sharpbacked puffer, *Canthigaster rivulatus* also produced mucous secretions that had a toxic effect in fish similar to that of *A. hispidus* mucus.

The endogenous toxin of the puffers, tetraedotoxin (or tetrodotoxin), has been reported to occur in the puffers examined and has probably confused the picture. The relationship between the endotoxin and the toxic mucous secretions has been investigated by W.H. Eger (1963).
CHAPTER 13

SITES OF OSTRACIN SECRETIONS IN THE BOXFISH

Foamy oral exudations were observed when freshly captured boxfish were taken out of water. Furthermore, "soapy" mucous secretions appeared at the base of the pectoral, anal and dorsal fins and at the caudal peduncle. Such mucous secretions were characteristic of all Hawaiian trunkfish examined and although "foaming of the mouth" has been reported by Gordon (1937) in Atlantic trunkfish, he attached no significance to it.

The definite correlation between foaminess of ostracin solutions and toxicity suggested a relationship between the foamy mucus and ostracin secretion. During the earliest stages of this investigation, both the oral foam and skin mucus were found to be ichthyotoxic, thus it became important to search for poisonous glands in the mouth and integument of the boxfish. The approach to this problem consisted of three parts: (1) to collect secretions from various portions of living boxfish and assay them for ichthyotoxic and hemolytic activity, (2) to extract various tissues with different solvents and assay the extracts, and (3) to conduct a thorough histological study of the probable glandular tissue.
ASSAY OF ORAL AND SKIN SECRETIONS

Materials and Methods

The oral foam was separately collected from 12 boxfish with an eyedropper while a cloth was wrapped around the fish's body just posterior to its eyes to prevent skin secretions from contaminating the sample. However, it was difficult to prevent skin secretions from being mixed with the oral exudations since the oral foam would cling to the skin of the lips and head of the boxfish. If the eyedropper was inserted into the boxfish's mouth the oral secretion was inhibited. In addition, the foamy mucus produced at the pectoral fin base could possibly be sucked into the branchial cavity through the opercular opening and eventually be exuded through the mouth.

The skin secretions were collected by wrapping the entire head of the boxfish with cloth and rinsing the skin with water, or pipetting off foamy mucus directly with an eyedropper. Samples were assayed for hemolytic and/or ichthyotoxic activity.

Results

The oral secretions of only 6 fish were demonstrated to be ichthyotoxic and in one instance a sample was non-toxic to fish but caused hemolysis of tuna erythrocytes. Ichthyotoxicity data on the oral secretions of 10 fish are presented in Table IX.

The skin secretions, collected by rinsing the boxfish
TABLE IX. Ichthyotoxicity of the Oral Secretions of Boxfish

<table>
<thead>
<tr>
<th>Total length of boxfish (mm)</th>
<th>Volume of diluted secretions</th>
<th>Volume assayed (ml)</th>
<th>Volume of assay sea water (ml)</th>
<th>Toxicity (mean survival in min. of 2 sailfin mollies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>5.6</td>
<td>1.0</td>
<td>100</td>
<td>60.0</td>
</tr>
<tr>
<td>105</td>
<td>11.0</td>
<td>2.0</td>
<td>100</td>
<td>50.0</td>
</tr>
<tr>
<td>120</td>
<td>6.8</td>
<td>2.0</td>
<td>50</td>
<td>86.0</td>
</tr>
<tr>
<td>113</td>
<td>2.2</td>
<td>2.2</td>
<td>50</td>
<td>non-toxic</td>
</tr>
<tr>
<td>95</td>
<td>6.4</td>
<td>6.4</td>
<td>50</td>
<td>non-toxic</td>
</tr>
<tr>
<td>95</td>
<td>6.3</td>
<td>6.3</td>
<td>50</td>
<td>27.0</td>
</tr>
<tr>
<td>117</td>
<td>2.4</td>
<td>2.4</td>
<td>50</td>
<td>36.0</td>
</tr>
<tr>
<td>69¹</td>
<td>1.6</td>
<td>0.1</td>
<td>15</td>
<td>non-toxic</td>
</tr>
<tr>
<td>75</td>
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<td>0.1</td>
<td>15</td>
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<tr>
<td>80</td>
<td>2.1</td>
<td>0.05</td>
<td>15</td>
<td>59.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>15</td>
<td>47.0</td>
</tr>
</tbody>
</table>

¹The samples from these fish were hemolytic; the others were not tested.
with water, were considerably more toxic than oral secretions and in the one case tested with RBC, caused strong agglutination of tuna erythrocytes prior to hemolysis. Ichthyotoxicity data on skin secretions are presented in Table X. The failure of the oral secretions tested to cause agglutination was probably due to ostracin concentration since agglutination occurs only at strongly ichthyotoxic concentrations (1:20,000).

The data indicate that the major secretion of ostracin occurs in the skin, and assuming that the oral exudations were not contaminated by skin secretions, ostracin is also produced in the oral cavity. The non-toxicity of the 2 skin samples may be due to the dose used in the assay, whereas the non-toxicity of the 4 oral secretions could mean that the skin secretions were actually responsible for the 6 toxic reactions obtained with oral foam.

EXTRACTION OF OSTRACIN FROM BOXFISH TISSUES

**Materials and Methods**

Various extraction techniques were tried and the extracts were assayed for ichthyotoxic and hemolytic activity. In all cases the tissues were washed thoroughly in distilled water to remove superficial secretions prior to extraction. The extraction methods and the tissues used are listed separately.

(1) A 110 mm long female boxfish was killed immediately
TABLE X. ICHTHYOTOXICITY OF BOXFISH SKIN SECRETIONS

<table>
<thead>
<tr>
<th>Total length of boxfish (mm)</th>
<th>Volume of diluted secretions (ml)</th>
<th>Volume assayed (ml)</th>
<th>Volume of assay sea water (ml)</th>
<th>Toxicity (mean survival in min. of 2 sailfin mollies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>12.0</td>
<td>2.0</td>
<td>100</td>
<td>10.5</td>
</tr>
<tr>
<td>105</td>
<td>15.0</td>
<td>3.0</td>
<td>100</td>
<td>20.5</td>
</tr>
<tr>
<td>...?</td>
<td>7.0</td>
<td>1.4</td>
<td>100</td>
<td>18.0</td>
</tr>
<tr>
<td>120</td>
<td>12.0</td>
<td>1.0</td>
<td>100</td>
<td>non-toxic</td>
</tr>
<tr>
<td>120</td>
<td>6.5</td>
<td>2.0</td>
<td>50</td>
<td>6.0</td>
</tr>
<tr>
<td>72(^1)</td>
<td>...?</td>
<td>...?</td>
<td>...?</td>
<td>...?</td>
</tr>
<tr>
<td>80</td>
<td>...?</td>
<td>0.05</td>
<td>15</td>
<td>185.0</td>
</tr>
<tr>
<td>116</td>
<td>10.0</td>
<td>0.50</td>
<td>15</td>
<td>non-toxic</td>
</tr>
</tbody>
</table>

\(^1\)The skin secretions of this fish were strongly hemolytic and agglutinative on tuna erythrocytes; the others were not tested on blood.
after capture by immersion in a strong solution of MS 222 (tricaine methanesulfonate) and the following tissues were dissected, extracted with sea water and centrifuged: skin, muscle, esophagus, stomach, intestine, liver, kidneys, ovaries, urinary bladder, and some subcarapace cheek "jelly". The supernatant fluid was assayed for ichthyotoxicity by the standard assay.

(2) The tissues of a large male boxfish which had been frozen were homogenized in a blender and extracted with hot ethanol (95%). The tissues extracted included the gall bladder, viscera, liver, anal and pectoral fin bases, gills, caudal skin, dorsal musculature, air bladder, lips, oral cavity epithelium, tongue and the subcarapace cheek "jelly". The ethanolic extract was assayed for ichthyotoxicity by the standard assay.

(3) The lips and the tail of a 90 mm long juvenile boxfish were severed at capture and frozen in sea water. After thawing they were extracted with boiling distilled water and the extract assayed for ichthyotoxicity.

(4) The lips of a 210 mm long male were severed at capture and both the lips and boxfish were frozen. After thawing, the lip skin was dissected from the jaws and both portions were extracted with hot water. The caudal skin and epidermal scrapings from the carapace were also extracted with hot water. All extracts were tested for ichthyotoxic and hemolytic activity.
(5) The lips of a 175 mm long female were severed, frozen and extracted with hot water and the extract assayed as in (4).

(6) The caudal skin, carapace, base of fins, lips and trunk muscles of a 175 mm long female boxfish were extracted with hot water followed by hot ethanol (70%) and the ethanol extract was dissolved in a citrate saline solution and assayed for hemolytic activity.

(7) The caudal and fin base skin of a 170 mm long male boxfish were extracted with acidified (HCl) hot water and the aqueous extract assayed as in (4).

(8) The lips of 4 large boxfish were severed, frozen and extracted with acidified (HCl) hot water and the aqueous extract was assayed as in (4).

(9) The lips, lateral and ventral carapace and caudal skin of a 110 mm freshly captured female boxfish killed by immersion in 35% isopropyl alcohol were cut up, extracted with sea water and assayed for ichthyotoxicity.

(10) The lips of 4 freshly captured boxfish were severed, extracted with citrate saline and assayed for hemolytic activity.

Results

All the extracts, with some exceptions in (3), (4), and (5) were non-toxic and non-hemolytic. Of the exceptions, extracts of the lips in (3) and (5) and the jaws in (4) were weakly ichthyotoxic. The jaws and lips in (4) and (5) were
strongly hemolytic but non-agglutinative. None of the skin extracts had any activity.

The results obtained in (6) cannot be considered valid since this fish had apparently been kept in captivity for a long time judging by its emaciated appearance and small liver.

**DISCUSSION**

The contradictory nature of the secretion and extraction results presents an intriguing enigma. A high yield of ostracin can be obtained from the stress secretions of living boxfish but practically no ostracin could be extracted from the suspected glandular tissues of dead boxfish. There are at least three possible explanations to this paradox: (1) the extraction techniques were inadequate or were causing detoxification; (2) the tissues extracted were not producers of ostracin; or (3) ostracin is activated by some unknown mechanism just prior to or during the actual secretion process.

The first explanation appears unlikely because the extraction techniques employed work well for other saponins and most water soluble, non-proteinacious toxins. Detoxification would only be possible in (7) and (8) where acidified water was used.

The second explanation must be carefully considered, but since all the major organs, with the exception of the nervous and skeletal systems, were extracted and assayed, it seems improbable that poison glands would be overlooked.
The foamy ostracin secretions appeared to form on all epidermal areas of living, distressed boxfish and highly toxic samples could be collected easily from the surface of the skin.

The third explanation appears at present to have the most merit. Since ostracin seems to be an active cytotoxin with high ichthyotoxic potency, it is possible that a detoxification mechanism exists in boxfish tissue wherein ostracin is stored in an inactive state. During secretion, ostracin might be activated by an enzyme or some other means as it is extruded. The boxfish, although highly resistant to ostracin, can be poisoned by this toxin (see Chapter 5) and hemolysis of boxfish erythrocytes by ostracin occurred in vitro (see Chapter 9). Thus, an accumulation of active toxin in the glandular tissues could have an adverse effect on the boxfish if ostracin secretory cells were damaged and ostracin leaked into the bloodstream.

The toxic extracts of the lips and jaws and the non-toxic skin extracts present another paradox since the oral secretions were only mildly toxic in comparison to skin secretions. However, these results could not be repeated and are probably artifacts caused by secretions clinging to the surface of these tissues.

Further discussion of these problems follows the histological study of the suspected glandular tissue.
CHAPTER 14

HISTOLOGY OF THE PRESUMED POISONOUS GLANDS
OF THE BOXFISH: MATERIALS AND METHODS

The evidence discussed in the preceding chapter, although contradictory and enigmatic, suggested that poisonous glands occurred in the oral cavity and skin of boxfish. A histological investigation was designed to test this hypothesis and to describe the structure of the glands and the nature of their secretions.

COLLECTION AND FIXATION OF TISSUES

All boxfish used for histological study were collected in Kewalo Basin and on the Ala Moana reef, Oahu. The fish were either killed immediately upon capture by immersing them in a fixative or by pithing, or they were left in a container with their toxic secretions until they died. The purpose was to obtain glandular tissue before and after the secretion process. Actually, it was almost impossible to be certain that secretion had not occurred prior to fixation since capturing and killing the boxfish were undoubtedly "distressing" and the degree of stress secretions remained an incalculable variable.

Selected tissues were dissected from the freshly killed boxfish and immediately immersed in an appropriate fixative. Fixatives used were: Bouin's fluid, 10% formalin in sea water, 10% formalin in 70% ethanol, a modified Gilson's
fluid (formaldehyde used instead of mercuric chloride: 10 ml 95% ethanol, 0.4 ml glacial acetic acid, 1.8 ml nitric acid, 5 ml formaldehyde and 88 ml distilled water, and Champy's fluid. Bouin's fluid was far superior to the other fixatives in preserving cellular structure and secretory products, and consequently became the standard fixative in this study.

Tissues selected for microscopical examination were:
the integument, which included skin from the caudal peduncle,
the folds at the junction of the carapace and caudal trunk,
the base of the pectoral, anal and dorsal fins, the dorsal, lateral and ventral carapace and the lips; the oral and buccal cavity, which included the jaws, the tongue, the palate to and including the esophagus and gills; the subcarapace cheek "jelly" and the opercular flap.

All Bouin-fixed tissues were washed and stored in 70% ethanol and certain tissues, e.g., the lips which contained the jaws and teeth, the carapace and the palate were decalcified in 3 changes of a 5% nitric acid solution for a week to 10 days.

DEHYDRATING, CLEARING, EMBEDDING, SECTIONING AND MOUNTING

Tissues were dehydrated with absolute ethanol and cleared with either cedarwood oil, methylbenzoate celloidin or toluene, with toluene giving the best results. Tissues were embedded in a medium grade paraffin (melting point 56 - 58° C) after infiltration for 3 to 5 hours.
The paraffin tissue blocks were sectioned at thicknesses of 4, 6, 8 and 10 μ. Serial sections (cross, sagittal or tangential) were prepared of all tissues. Most skin sections were cut at 6 μ, whereas other tissues (lip, mouth and carapace) were cut at 8 to 10 μ.

The paraffin ribbons were affixed to microscopical slides with an albumen fixative diluted with distilled water (2 drops albumen to 20 ml water).

STAINS AND STAINING
(1) Harris hematoxylin. This nuclear stain was used on all sections to determine general histological structure. Hematoxylin sections were counterstained with eosin or triosin.
(2) Heidenhain's iron hematoxylin. Allen's modification of this stain was used with and without counterstains (eosin or triosin). Finer cytological detail was possible with this stain than with Harris hematoxylin.
(3) Mallory's triple stain. This connective tissue stain was useful in differentiating the integumentary sections and glandular secretions. Brilliant staining occurred after Bouin's fixation.
(4) Gomori's aldehyde fuchsir. This stain was developed by Gomori (1950a) for elastic tissues. He states that the staining properties of this dye are almost unique in that it has been found to stain special types of secretory
cells such as mast cells, Beta cells of pancreas and certain basophils of the anterior pituitary, and will also stain certain types of mucin and elastic fibers of all tissues. This dye was used with a trichrome stain originally developed by Gomori (1950b) and later modified by various workers as follows: sections first mordanted in a 4% phosphotungstic acid — 1% phosphomolybdic acid solution for 10 minutes and stained for 1 hour in an aqueous trichrome stain containing 0.4% fast green, 1% orange G, 0.5% chromotrope 2R and 0.28% acetic acid.

(5) Periodic acid leucefuchsin Schiff method (PAS).

The PAS procedure recommended by Conn et al. (1960) was followed, although the nuclear and counterstains were not used. This stain was employed to characterize epithelial mucins and other cytoplasmic inclusions.

(6) Toluidine blue. This is a metachromatic basis dye which stains basophilic substances, including many acidic mucins, and was used to characterize epithelial mucins. Both aqueous and ethanolic solutions were used following the procedures listed in Pearse (1960, pg. 834).

(7) Dresbach’s mucicarmine method. This method was used to demonstrate epithelial mucins.

(8) Sudan Black B. This stain was used after fixation in Champy’s fluid to demonstrate lipids in the cytoplasm of various glandular cells in paraffin sections (see Pearse, 1960, pg. 850).
CHAPTER 15

HISTOLOGY OF BOXFISH SKIN

Teleost skin typically consists of an epidermis of stratified epithelium containing mucous cells, sensory cells and ordinary squamous or cuboidal epidermal cells, and a dermis containing scales of various types in different species. There are numerous variations of this basic pattern and a few curious specializations.

In some families of teleosts, serous gland cells and specialized secretory cells of a dubious function occur in the epidermis. Rabl (1931) has listed a few families of bony fishes, widely separated phylogenetically, that contain in their epidermis special secretory cells, called "club cells" because of their characteristic shape, which are thought to be modified serous gland cells. Club cells are characterized by the discharging of their secretion while in the middle layers of the epidermis. The resulting degenerate cell is often pushed onto the surface of the epidermis and discarded (Andrew, 1959).

There is an extensive literature on the distribution and structure of these cells in the bony fishes (see Rabl, 1931) and many speculations on their function. Because these club cells are also associated with the poisonous glands of venomous fishes such as the Scorpaenidae (scorpion fishes), Trachinidae (weever fishes), Synancejidae (stonefishes),
Batrachoididae (toadfishes), and many others, they are also suspected of producing a poisonous defensive secretion in the non-venomous fishes such as the Cyprinidae (minnows), Anguillidae (eels), and Gadidae (cods). Counterparts of these cells are the cells of Leydig which form the poisonous secretions in many amphibians (Andrew, 1959).

No literature was available on the histology of the skin of the Ostraciontidae although Rosen (1913) described the integument of trunkfish, but this work was chiefly concerned with the dermal carapace and only scant mention was made of the epidermis.

GENERAL MORPHOLOGY OF THE SKIN

Boxfish skin contains both hard and soft parts. The "hard" dermal carapace extends throughout the body trunk and the head. The "soft" skin is found covering only the fleshy lips, the base of the fins, and the caudal peduncle. There are no spines in this species and the integument feels smooth and slimy to the touch. Inward folding of the skin occurs at the base of the fins and at the junction of the trunk carapace with the caudal peduncle. In these areas the foamy mucous secretions were most conspicuous, probably due to the movement of fins lathering the secretions.

When a live, distressed boxfish was immersed in a fixative (Bouin's fluid or 10% formalin), a thick, whitish mucoid layer would congeal over the epidermis (see Figure 13).
Figure 12. Surface view of the mid-lateral carapace of the boxfish (formalin fixation). X40.

Figure 13. Surface view of the lateral carapace behind the pectoral fin showing the thick, congealed mucoid coating (formalin fixation). X40.
This layer was thickest on the carapace behind the pectoral fin, although this mucoid coating was of sufficient thickness on other portions of skin so that it could be peeled off intact as one peels off sunburned skin.

The epidermis on the lips, parts of the carapace and caudal peduncle is partitioned by dermal septa into reticulated shallow "pockets". The surface of these "pockets" can be seen in Figures 12 and 13. In other sections of the epidermis, such as around the fin bases and parts of the carapace, the "pockets" are indistinct, but dermal ridges, grooves and protuberances cause irregularities in the epidermis and effectively increase its surface.

No axillary glands or other accessory skin glands were found in a macroscopic examination. Nor could any subcarapace pockets of "jelly" be found behind the gill opening as was noted by Brown in an Atlantic trunkfish (see Chapter 1, pg. 4).

STRUCTURE OF BOXFISH EPIDERMIS

The epidermis consists of stratified squamous epithelium and is sharply delineated from the dermis by a border of melanophores. At least 3 distinct cell types can be recognized in the epidermal layer:

(1) Epidermal cells. These are the unspecialized supporting cells which may be considered as forming the "matrix" of the epidermis. They are distinguished by their large, usually spherical, nuclei which measure about 4 μ in diameter.
The polyhedral cell outlines are irregular and the cytoplasmic portion of the cell is quite small in comparison to the large nucleus. In the basal layer of the epidermis the nuclei are often fusiform and the cells are columnar-shaped, whereas near the surface and in the intermediate layers polyhedral cells with spherical nuclei prevail. On the surface of the epidermis occurs a single layer of squamous cells with serrated edges, forming the external boundary of the stratified epithelium.

(2) Mucous cells. These cells, about 12 - 16 μ in diameter, are only found at or near the surface of the epithelium. Their nuclei are spindle-shaped and peripheral and the intact cells were always full of mucus. On some sections "ruptured" cells could be seen discharging their mucous content (see Figure 14). Evidently the cell membrane collapses and the mucous contents are eliminated during the process of secretion, i.e., the secretion appears to be of the merocrine type.

(3) Club cells. These large (25 to 50 μ), variable-shaped "serous" cells were the most conspicuous cells in the epidermis. They are classified as "club cells" because they were found secreting their products while in the middle layers of the epidermis, and they resemble the so-called "club cells" of other teleosts in structure and in the appearance of "secretory products". Their secretion also appears to be of the merocrine type.
Figure 14. A mucous cell in the process of extrusion. Heidenhain's iron hematoxylin. X1000.

Figure 15. A stage I club cell. Note the large spherical nucleus with prominent nucleolus and fine granular cytoplasm. Heidenhain's iron hematoxylin. X1000.
These club cells may be seen in various stages of development. The description of these proposed stages, based upon studies of Bouin-fixed, Heidenhain's iron hematoxylin stained sections cut at 6 μ is as follows:

(a) In the early developmental stage (I) a large (7 to 8 μ) spherical nucleus may be seen in the center of the cell. The cytoplasm consists of very fine granular material which stains a uniform gray. Such cells were usually found in the basal layers of the epidermis (see Figure 15).

(b) In about the same position in the epidermis are cells in the second stage of development (II), (see Figure 16). These cells are characterized by coarse, deeply stained granules forming among the fine granular cytoplasm. At this stage orientation of the cell into apical and basal poles is obvious. The spherical nucleus can now be seen at the basal end, apparently having been forced there by the accumulating secretion products.

(c) In the third developmental stage (III) the coarse granules accumulate at the apical pole and a thick cap of homogenous, colloidal secretion forms at the apex and tapers down along both sides (see Figure 17). At this stage the cell can be seen discharging the homogenous secretion while still in the middle layers of the epidermis. The nucleus
Figure 16. A stage II club cell. Note the formation of coarse granules and the polar orientation of the cell. Heidenhain's iron hematoxylin. X1000.

Figure 17. A stage III club cell showing the formation of the apical cap. Heidenhain's iron hematoxylin. X1000.
is somewhat flattened against the basal end of the cell and is often difficult to recognize.

(d) The fourth developmental stage (IV) is characterized by a large globule of homogenous, colloidal secretion. The coarse granules are absent or are incorporated within the globule. These cells are usually found closer to the surface than those of the preceding stages and the homogenous secretion often may be seen being extruded as the cell wall ruptures. The oblong, somewhat flattened nucleus is found along the periphery of the cell (see Figure 18).

(e) Cells in the fifth developmental stage (V) are found closest to the surface and are somewhat reduced in size. They appear to be in a state of involution. The globular secretion is reduced in size and appears more spherical. The greatly flattened nucleus is still evident (see Figure 19).

Size and position of club cells are not necessarily indicative of developmental stages, as these criteria are variable. The most important criterion is the type of cell inclusions, or secretory products present.

Many of the club cells appear as empty vacuoles, and even the cells containing secretions have extensive open spaces. The latter is probably a fixation artifact, as the flattened peripheral nuclei of the club cells suggest that the cells are full. The empty vacuoles indicate that either
Figure 18. A stage IV club cell showing the homogenous, colloidal secretion and the flattened nuclei. Heidenhain's iron hematoxylin. X1000.

Figure 19. An early stage V club cell not yet in the process of involution. Heidenhain's iron hematoxylin. X1000.
secretion has already taken place or the secretory material was lost in sectioning.

A composite drawing of a cross section of boxfish skin showing all the cell types and stages of club cell development is presented in Figure 20.

FORMATION OF POISON SKIN GLANDS

In the skin areas where shallow pockets or grooves are formed by dermal septa (see Figures 12 and 13), groups of 3 - 5 club cells were found in the depressions between the septa. The apices of the club cells converge toward the center of the pocket and discharge their secretions into a common groove or pseudo-duct. Figure 21 shows 2 club cell "glands" and Figure 22 shows a pseudo-duct through which the secretions pass.

Multicellular skin glands, though rare in fishes, do occur as poison glands in certain elasmobranchs and teleosts and as phosphorescent organs in some bathypelagic fish (Van Oosten, 1957). Mucous glands in fish skin are unicellular and when mature, line the surface of the epidermis, whereas in amphibians there occur true multicellular glands extending into the dermis. The presence of primitive multicellular glands in boxfish epidermis marks an advance over most fishes, and since these glands consist of club cells, suggests a specialized function.

THE DISTRIBUTION OF MUCOUS AND CLUB CELLS IN THE EPIDERMIS

Mucous and club cells were found in all skin sections,
Figure 20. Drawing of a caudal skin section of *O. lentiginosus*.

Ep: epidermis  
bm: basement membrane  
De: dermis  
mI: melanophore  
cmp: mucus cell  
nc: nuclei of club cell  
ms: mucus secretion  
epc: epidermal cells  
I-V: stages of club cells  
hs: homogenous secretion of club cell  
gs: granular secretion of club cell
Figure 21. Club cell glands in the caudal skin of *O. lentiginosus*. Note the dermal septum separating the glands and the dark secretory product being extruded by the gland on the right. Heidenhain's iron hematoxylin. X440.
Figure 22. A club cell gland clearly showing the pseudo-duct. Harris hematoxylin-triosin. X400.
including those of the fin bases, lips and the carapace. The epidermis covering the tough dermal carapace was surprisingly rich with both mucous and club cells. As a general rule, naked or sparsely scaled fish are more richly provided with both numbers and kinds of mucous cells than the well scaled teleosts, and some investigators have postulated an inverse relationship between squamation and abundance and type of mucous cells (Van Oosten, 1957). Their reasoning is that the skin's protective function is assumed by the increased number of mucous cells in poorly scaled fish. The presence of such a glandular epidermis over the boxfish's hard carapace contradicts this generalization.

Club cell "glands" were found only in sections of the lateral carapace where epidermal pockets were observed superficially. However, not enough sections were examined to conclude that they are absent in the ventral and dorsal carapace. These "glands" were also found in the lip skin but were not encountered in sections of the dorsal, anal and pectoral fin bases.

STAINING AFFINITIES OF MUCOUS AND CLUB CELLS

The descriptions of staining properties pertain to skin sections fixed in Bouin's fluid unless otherwise specified.

Harris Hematoxylin and Triosin (or Eosin)

The colloidal nature of the club cell secretion
(stage III - V) was evident in these sections. Fine granules within the otherwise homogenous secretion in stages II and III stained only weakly in Harris hematoxylin and were not as conspicuous as they were in Heidenhain's iron hematoxylin preparations of the same tissue section. The homogenous secretion in club cells, stage III - V, stained red with trichosin or eosin and pale lavender in sections without these counterstains.

The mucous cells were stained a pale pink by trichosin or eosin and fine granular material could be distinguished.

Figure 23 shows a typical Harris hematoxylin-trichosin section of the caudal skin.

**Mallory's Triple Stain**

The club cell (III - V) secretions stained yellow-orange to a brilliant red and the sparse fine granules were still evident within the globule. Some of the club cell globules (IV) were bicolored, with the basal portion staining blue, and secretions of stage V cells often staining pale orange, red or blue. The color of the coarse granules in stage II and III appeared orange to dark red or purple, depending upon the thickness of the section and degree of destaining.

The mucous cells stained light blue and their granular content was evident.

The nuclei of all cells stained pale orange and were difficult to see, thus limiting the utility of this stain.
Figure 23. A Harris hematoxylin-triosin section of the caudal skin of O. lentiginosus. X400.

Figure 24. Mallory's triple stain section of the caudal skin. Note the blue staining mucous cells and the red homogenous secretions of the club cells. X400.
Clusters of coarse red granules were found for the first time in the basal layers of the epidermis. These granules were encountered in a few sections in both the epidermis and among the dermal connective tissue. Some resembled the nuclei of erythrocytes in size, structure and staining properties, whereas others appeared as disintegrated nuclei. Possibly, hemolysis of these erythrocytes by ostracin occurred during capture and handling prior to fixation. This would corroborate the in vitro hemolysis of boxfish erythrocytes by semi-pure ostracin.

Figure 24 depicts a typical caudal skin section with these red granules, and Figures 25 and 26 show extrusion of the secretory products of stages III and IV stained with Mallory's triple stain.

_Gomori's Aldehyde Fuchsin and Trichrome Counterstain_

The coarse granules in club cell stages II and III and the granules in the mucous cells were stained purple by aldehyde fuchsin. The homogenous secretions in III - V were stained orange, red and green by the counterstain and bicolored (green basal and red-orange apical portions) globules were common. Green-colored globules (IV and V) were much more numerous than the corresponding blue from Mallory stained sections although the colloidal secretion in stage III always stained red or orange in both stains.

The coarse red granules (believed to be disintegrated
Figure 25. A Mallory's triple stain caudal skin section showing the mid-epidermis extrusion of a stage III club cell. X400.

Figure 26. A Mallory's triple stain caudal skin section showing extrusion by stages IV and V club cells. X400.
Erythrocytes observed in Mallory sections were stained red-orange by the trichrome counterstain. The nuclei of all cells were weakly stained by the counterstain and an intercellular matrix interwoven among the epidermal cells was stained light green.

A caudal skin section stained with these dyes is shown in Figure 27.

The Periodic Acid Leucofuchsin Schiff's Method (PAS)

The PAS method was used to demonstrate glyco- and mucoproteins after digestion with amylase (from saliva). The only positive PAS substance in skin sections were the granules in the mucous cells which were strongly stained a deep red-violet color. The globular secretions in club cells (stage III - V) were stained pale pink, only slightly darker than the epidermal cells, indicating a negative reaction, or absence of glyco- and/or mucoproteins.

A skin section stained by the PAS method is shown in Figure 28.

Toluidine Blue

The homogenous secretions in club cells III - V were orthochromatically stained a deep aqua blue by this dye.

All nuclei were stained the color of the dye whereas the mucous cells were unstained.

Dresbach's Mucicarmine Method

In the epidermis only the homogenous secretion in the club cells was stained red by this commonly used mucous
Figure 27. A caudal skin section stained with Gomori's aldehyde fuchsin-trichrome stain. Note the aldehyde fuchsin positive granules in the mucous cells and in a stage III club cell and the "hemolyzed erythrocytes" in the basal layers. X400.

Figure 28. A caudal skin section stained with the PAS reagent. Note the PAS+ mucous cells. X400.
stain, but the connective tissue matrix in the dermis appeared pink. This method was recommended for epithelial and connective tissue mucins in general.

**Sudan Black B**

The homogenous club cell secretions were stained weakly with this dye in sections fixed with Chamy's fluid, but were not, or only weakly, stained in Bouin-fixed material. This suggests that lipids were present in the secretory materials but only in insignificant amounts.

**Discussion**

The staining affinities of the secretory materials in the epidermis corroborate that the mucous and club cells are distinct cell types with different secretory material and the developmental stages of the club cells, as described from iron hematoxylin sections, are in proper sequence.

There are apparent contradictions in the mucous staining properties of both glandular cells. The mucous cells stained positively with Mallory's triple (blue), PAS (red-violet), and aldehyde fuchsin (purple), indicating their mucoid nature. However, the club cell homogenous secretions were stained by toluidine blue and mucicarmine which are also mucous stains.

Thus, it appears that both cells contain mucins but of distinctly different types. The PAS positive reaction in the mucous cells shows that muco- and/or glycoproteins
are present and the negative PAS reaction in the club cells demonstrates their absence. By process of elimination, the other common epithelial mucins are the mucopolysaccharides, which are PAS negative substances and toluidine blue (β metachromasia) positive. However, at this point, the exact nature of the mucoid component is irrelevant. The important consideration is which cell, the mucous cell or the club cell, is responsible for estracin secretion?
CHAPTER 16
HISTOLOGY OF THE LABIAL GLANDS

As previously mentioned (Chapter 13), the oral secretions of boxfish contained an ichthyotoxic, hemolytic substance, although the skin secretions appeared to be the major source of ostracin. Histological sections were prepared of the entire lips, including the jaws and epithelial lining of the buccal cavity to and including, the esophagus. The more striking features of the histology of these regions are presented in this chapter.

GENERAL MORPHOLOGY

The most conspicuous structures in the anterior portion of the buccal cavity of boxfish are the prominent labial villi attached to the ventral floor of the vestibular region between the external lips and the teeth. A drawing of a median sagittal section of the lips of a female boxfish (Figure 29) clearly shows the position of these villi in the buccal cavity.

HISTOLOGICAL STRUCTURE OF THE LABIAL VILLI

Cross and sagittal sections (8 - 10 μ) of the lips revealed that the labial villi were extensions of dermal connective tissue lined with a mucous membrane composed of stratified cuboidal epithelium.

The epithelial cells were nearly uniform in size
Figure 29. Drawing of a sagittal section of the lips of a boxfish showing the position of the labial villi glands.

Lv: labial villi
Lpg: labial pocket glands
Le: labial epidermis
Tb: tooth bud
Ov: oral valve
Bc: buccal cavity
Ld: labial dermis
T: tooth
Tg: tongue
(about 12 μ) and occurred in great clusters at the distal ends of the villi. The cells appeared empty in iron hematoxylin sections, except for irregularly shaped spherical nuclei. Serrated edges were found on the peripheral cells. These "labial" cells were further characterized by their staining affinities with stains used on the skin sections in Chapter 15.

**Harris Hematoxylin and Triosin (or Eosin)**

Labial cells appeared the same as they did in iron hematoxylin sections. The spherical nuclei, agranular cytoplasm and serrated edges were evident.

**Mallory's Triple Stain**

The cytoplasm appeared clear in this stain also. The nuclei stained pale red and the dermal supporting framework deep blue (see Figure 30).

**Gomori Aldehyde Fuchsin and Trichrome Counterstain**

Dramatic results were obtained by staining the labial cells with aldehyde fuchsin. Fine and coarse purple granules were abundant in the cytoplasm. The distribution and relative density of these granules were more or less uniform from cell to cell and none of the cells was empty (see Figure 31).

**Periodic Acid Leucofuchsin Schiff's Method (PAS)**

PAS positive granules were demonstrated in the labial cells, however fewer granules were shown in these sections than in aldehyde fuchsin stained sections (see Figure 32).
Figure 30. Labial glands stained with Mallory's triple stain. Note the clear cytoplasm and red nuclei. X400.

Figure 31. Labial glands stained with Gomori's aldehyde fuchsin-trichrome stain. The positive aldehyde fuchsin granules are clearly visible. X400.

Figure 32. Labial villi stained with the PAS reagent. Note the positive PAS granules in the labial cells. X400.
Toluidine Blue

This metachromatic dye did not stain the labial cells.

The staining reactions of the contents of the labial cells were very similar to those of the mucous cells of the skin. The mucous cells however, stained more strongly in these dyes than the labial cells. This may be due to the relative amount of granular material in the cells rather than different types of reactants.

DISTRIBUTION OF THE LABIAL GLANDS

The labial villi were found to be only a portion of an extensive elaboration of the mucous membrane of the oral cavity into what will be referred to as "labial glands". The complexity of this glandular system may be sufficient to classify the labial glands as compound acinous glands.

In Ostracion, the labial glands consisted not only of dorsal and ventral villi but "glandular pockets" extending deeply into the dermis of the lips through infolding of the lip epidermis, therefore effectively forming a duct emptying outside the buccal cavity (see Figure 33).

These "pockets" are found in both the dorsal and ventral portions of the lip where they extend in an anterior to posterior direction with the duct opening at the surface of the lip epidermis. Other such pockets occur deep into the epithelial lining of the buccal cavity with ducts
Figure 33. A sagittal section of the lips showing labial pocket glands and their pseudo-ducts. Mallory's triple stain. X100.

Figure 34. A cross section of the lips showing the labial pocket glands. Gomori's aldehyde fuchsin-trichrome stain. X100.
opening into the oral cavity behind the teeth. See Figure 34 for a cross section of these dermal pockets.

The labial villi are contained in large pockets formed by connective tissue septa, both above the dorsal row of teeth and below the ventral row, with each pocket opposite each tooth. These will be referred to as the "dental pockets". Figure 35 is a drawing of a cross section of the lips and buccal cavity cut at the level of the dorsal row of teeth. The dermal pockets, and the villi within the dental pockets are shown.

The labial gland cells extend throughout the mucous membrane of the buccal cavity and are found lining the folds and papillae of the palate but do not occur in the esophagus.

STRUCTURE OF THE LIP SKIN AND PROBABLE ORIGIN OF THE LABIAL GLANDS

The epidermis of the lips is considerably thicker and more compact than that of the rest of the skin. The epidermal, or supporting cells, predominate although mucous and club cells are found in the upper layers, and in "glandular pockets", similar to those found in caudal skin sections, with distinct pseudo-ducts (see Figure 36).

In the anterior portions of lip sections extensive folding and invagination of the epidermis was evident. Some "glandular pockets" extended deeply into the lips and cross sections through these dermal pockets revealed
Figure 35. Drawing of a cross section of the mouth through the dorsal teeth showing the arrangement of labial villi around the teeth.

Lv: labial villi
Lpg: labial pocket glands
Lsg: labial skin glands
Oc: oral cavity
D: dorsal
V: ventral
T: teeth
Figure 36. Lip skin showing mucous and club cells among the thick layer of epidermal cells. Gomori's aldehyde fuchsin-trichrome. X400.

Figures 37 and 38. Labial epidermal folds showing the transition from the skin gland cells to labial cells. (Left: Gomori's aldehyde fuchsin trichrome stain. X400. Right: Mallory's triple stain. X250.)
mucous cells, club cells and labial cells. A transition of cellular types occurred, with the mucous and club cells being found in the more superficial folds and labial cells being found exclusively in the deep pockets, but intermediate regions contained all 3 types of cells (see Figures 37 and 38) which suggested that the labial glands are formed by an invagination of the lip epidermis.

Although the staining properties of the labial cells are similar to those of the skin mucous cells, there are enough differences to conclude that these cells are a distinct type: (1) the labial cells possibly have a merocrine secretion but no cells were observed in the process of extrusion, (2) serrated edges were found on some labial cells, but could not be demonstrated in mucous cells, (3) the nucleus of mucous cells was always peripheral and spindle-shaped like that of typical mucous cells, whereas the labial cell nuclei were irregularly-shaped spheroids, (4) mucous cells stained blue in Mallory's triple stain but the labial cells remained clear, (5) mucous cells were strongly PAS positive but labial cells contained only a few PAS positive granules, and (6) granules of both cells stained purple with aldehyde fuchsin but staining was much more intense in the mucous cells.

**DISCUSSION**

Compound glands rarely occur in the buccal cavity of fishes, although Andrew (1959) notes multicellular glands
in the lungfish *Protopterus annectans*, Nishida (1954) in adult salmon *Onocorhynchus keta* and Monod (1950) in the characin *Alestes* sp. The function of such glands is unknown and true salivary glands are thought to be absent in fishes.

The multicellular labial glands of the mucous membrane of boxfish are prominent and extensive enough to justify consideration of them as poisonous glands. An ichthyotoxin hemolysin has been found in the oral secretions collected from the region near the labial villi (see Chapter 13). On the other hand, the skin secretions were considerably more toxic than the oral ones and no active substances could be extracted from the skin or lips of dead boxfish. Furthermore, the labial and skin glands are histologically distinct, and the cytomorphology and histochemical properties of the secretory cells of the gland cells are different. See Table XI for a summary of the staining reactions of the secretory material within the gland cells.

Such evidence suggests that 2 or more distinct secretions containing different toxins are occurring, but this has not yet been demonstrated. Because of difficulties met in extracting ostracin from the skin and the small yield of toxin from lip extracts, it has not been possible to characterize chemically the respective toxins. Nevertheless, highly toxic skin secretions could be collected from distressed boxfish from which the lips, containing the labial glands, were completely severed. Assuming that ostracin
<table>
<thead>
<tr>
<th>STAINS</th>
<th>MUCOUS CELLS</th>
<th>CLUB CELLS</th>
<th>LABIAL CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>homogenous secretion</td>
<td>coarse granules</td>
<td></td>
</tr>
<tr>
<td>Harris hematoxylin triosin</td>
<td>light red</td>
<td>red</td>
<td>pale pink</td>
</tr>
<tr>
<td>Heidenhain's iron hematoxylin</td>
<td>clear</td>
<td>black</td>
<td>black</td>
</tr>
<tr>
<td>Mallory's triple stain</td>
<td>blue</td>
<td>orange, red or blue</td>
<td>dark red to violet</td>
</tr>
<tr>
<td>Gomori's aldehyde fuchsin-trichrome</td>
<td>deep purple</td>
<td>orange, red or green</td>
<td>deep purple</td>
</tr>
<tr>
<td>PAS</td>
<td>deep red-violet</td>
<td>pale pink</td>
<td>pale pink</td>
</tr>
<tr>
<td>Toluidine Blue</td>
<td>clear</td>
<td>deep aqua</td>
<td>blue to light aqua</td>
</tr>
<tr>
<td>Mucicarmine</td>
<td>clear</td>
<td>pink to red</td>
<td>pink</td>
</tr>
<tr>
<td>Sudan Black B</td>
<td>clear</td>
<td>gray</td>
<td>very light gray</td>
</tr>
</tbody>
</table>
secretion has a protective function, this indicates that the labial glands play a minor role in the total ostracin secretion by distressed boxfish. The position of the glandular labial villi and pockets in the buccal cavity suggests a possible feeding, rather than defensive, function.
CHAPTER 17

HISTOLOGICAL AND HISTOCHEMICAL EVIDENCE OF OSTRACIN SECRETION

The experimental evidence of the site of ostracin secretion and the description of the histological structure of the skin and labial glands clearly suggest that ostracin is being secreted by epidermal glands. However, the anomalies obtained in tissue extracts, together with the establishment of 3 distinct gland cells, make it difficult to ascertain the precise source of ostracin. Additional studies attempting to resolve this problem are presented in this chapter.

HISTOLOGICAL CHANGES IN THE LABIAL AND SKIN GLANDS OF CAPTIVE BOXFISH

In Chapter 7 it was shown that boxfish in captivity would retain their ability to secrete ostracin. However, poorly fed or starving fish secreted very little ostracin under stress. Thus, the glandular tissues of captive boxfish were fixed, sectioned and stained to correlate the reduction in toxicity with changes in histological structure.

One 81 mm long juvenile boxfish was kept in an aquarium for 32 days and given a subminimal diet of Purina trout food and frozen brine shrimp. On the thirty-second day the stress secretions of this fish were collected in 7.2 ml of tap water until death of the boxfish and assayed for ichthyotoxicity with 2 sailfin mollies in 100 ml of sea water. The results were as follows:
<table>
<thead>
<tr>
<th>Vol. assayed of the 7.2 ml rinse</th>
<th>Mean survival time of assay fish (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>non-toxic</td>
</tr>
<tr>
<td>1.0</td>
<td>non-toxic</td>
</tr>
<tr>
<td>3.0</td>
<td>102.0</td>
</tr>
</tbody>
</table>

Calculation of the total amount of ostracin from the dose-response curve (Figure 8) shows that less than 1 mg of ostracin was present in the secretions of this fish. This represents a considerable reduction in ostracin yield from freshly captured boxfish.

The lips and portions of the caudal skin of this fish were fixed in Bouin's and processed for histological examination. Results of this examination are as follows:

The Epidermis

There was a marked change in the epidermis. Both mucous and club cells were greatly reduced in number and many sections of the skin were completely devoid of these cells, consisting only of undifferentiated epidermal cells (see Figure 39). The mucous cells, though few in number, were full of mucus, and the club cells were either empty or contained coarse granules which were stained purple by aldehyde fuchsin. Only a very few club cells contained a homogenous secretion.

Another captive fish, a 115 mm long male, lived for 48 days in an aquarium. When this boxfish became moribund its tissues were fixed in Bouin's and processed for histological
Figure 39. The skin of a starved boxfish (32 days in captivity). Note the lack of gland cells and the proliferation of epidermal cells. Harris hematoxylin. X400.
examination. The skin sections of this fish resembled those of the captive juvenile fish in their scarcity of mucous and club cells. This fish had been feeding better than the first but was so emaciated and weak when it died that no stress secretions were collected.

The numbers of mucous and club cells in the caudal epidermis of both boxfish kept in captivity were counted and compared with numbers of these cells in freshly captured boxfish. The counts were averaged per linear mm from several skin sections (6 μ) and, although variations in gland cell numbers can be expected to depend upon the section chosen, general information on the relative abundance of these cells can be obtained. The results are tabulated in Table XII.

The data indicate that there is a greater proportionate reduction in the number of club cells as compared to mucous cells in boxfish held in captivity. The ratio of mucous cells to club cells is even greater when a skin section containing "glandular pockets" is examined, however such a section cannot be used for a direct comparison since the captive boxfish skin sections did not contain "glandular pockets". Nevertheless, these data could be interpreted to suggest that the club cells are responsible for ostracin secretion.

Labial Glands

A great reduction in the number of labial cells
TABLE XII. RELATIVE NUMBERS OF MUCOUS AND CLUB CELLS IN THE CAUDAL EPIDERMIS OF BOXFISH KEPT IN CAPTIVITY AND FRESHLY CAPTURED

<table>
<thead>
<tr>
<th>Caudal skin section (6 μ)</th>
<th>Mean no. of mucous cells per mm epidermis</th>
<th>Mean no. of club cells per mm epidermis</th>
<th>Approx. ratio of mucous to club cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boxfish in captivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(glandular &quot;pockets&quot; absont)¹</td>
<td>5.7</td>
<td>10.3</td>
<td>(1:2)</td>
</tr>
<tr>
<td>Freshly captured boxfish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(glandular &quot;pockets&quot; absent)²</td>
<td>12.6</td>
<td>45.0</td>
<td>(1:4)</td>
</tr>
<tr>
<td>(glandular &quot;pockets&quot; present)³</td>
<td>9.9</td>
<td>61.6</td>
<td>(1:6)</td>
</tr>
</tbody>
</table>

¹ Average of 6 sections (total 19.7 mm) from 2 boxfish, 32 and 48 days in captivity.

² Average of 5 sections (total 16.6 mm) from 2 boxfish, freshly captured.

³ Average of 2 sections (total 8.6 mm) of 1 boxfish freshly captured.
occurred in both boxfish kept in captivity. A great proliferation of epidermal cells almost completely occluded the lumen of the labial "pockets" (see Figures 40 and 41). Often only scattered cells or a single row of labial cells lined the inside of these "pockets".

The labial cells along the villi were also reduced in numbers and often only a single row of these cells occurred, but this reduction was not as great as that which occurred in the labial "pockets".

The "glandular pockets" in the lip skin consisted almost entirely of epidermal cells. Only a scattered few club and mucous cells were seen in the fish held in captivity for 32 days, however there was little change from normal in the lip epidermal glands of the fish held 48 days in captivity. The former fish was a juvenile and the latter an adult male, possibly accounting for the variability in the lip skin.

GLANDULAR HISTOLOGY OF STRESSED VERSUS NON-STRESSED BOXFISH

Boxfish, as mentioned previously, were either rapidly killed by immersion in a fixative or left to die in their mucous secretions. The purpose was to obtain glandular tissue prior to and after secretion of ostradin. A cursory examination of the skin sections of such stressed and non-stressed boxfish showed that there appeared to be more empty club cells in the former. However, the skin sections
Figure 40. Labial pocket glands of a starved boxfish (48 days in captivity). Mallory's triple stain. X100.

Figure 41. Labial pocket glands of the same fish stained with Gomori's aldehyde fuchsian-trichrome. Note the few labial cells among the proliferating epidermal cells. X400.
used were not comparable and the degree of stress was not controlled.

In an attempt to control the amount of stress that boxfish were subjected to, 2 juvenile boxfish (62 and 66 mm long) were carefully captured with a dipnet on Ala Moana reef and immediately transferred into a 20 gallon bucket sealed with a lid. The 2 fish had been observed swimming together and both were easily captured within a few minutes. They were handled as little as necessary and were not unduly excited.

Both fish were kept in a 35 gallon aquarium for 3 days. On the third day one of the boxfish (62 mm), chosen at random, was quickly netted and rapidly killed by immersion in Bouin's fluid. The other (66 mm) was placed in 1 liter of sea water and kept there for 2 hours. Air was periodically bubbled through the water and the sides of the container tapped to disturb the boxfish and cause a stress situation. A small puffer (Canthigaster jactator) was placed in with the boxfish as an assay for ostracin. The puffer died after 28 minutes, indicating that ostracin had been secreted by the distressed boxfish. When the boxfish appeared weakened, it was killed by immersion in Bouin's fluid. This fish had not been handled during the induced stress experiment since direct pressure on the epidermis might have caused excessive secretion of the glands.

The glandular tissues (fixed in Bouin's) of both fish
were processed for histological examination.

**The Epidermis**

Caudal skin sections of both fish were stained with Mallory's and with Gomori's aldehyde fuchsin-trichrome. Mucous and club cells (stages II - III with granules, IV - V with colloidal globules and empty cells) were counted and their number per linear mm calculated. The results are presented in Table XIII.

The data clearly indicated that empty club cells are a poor criterion for comparing stressed and non-stressed boxfish, although the relative ratios of the club cell developmental stages may be suggestive of the actual secretory cycle. However, firm conclusions cannot be made since information is lacking on the timing of the secretory cycle. The differences in club cell counts noted in the "after stress" fish appear to be insignificant and the difference in the number of mucous cells seems to argue against this cell secreting ostracin.

**The Labial Glands**

The labial glands of both fish, stained with aldehyde fuchsin to demonstrate their granular content, appeared similar in granular density and no significant differences were detected.

Thus, the results of this controlled stress experiment were negative. Apparently the "stress" conditions imposed upon the "after" fish were not great enough to cause maximal secretory activity.
TABLE XIII. COMPARISON OF NUMBERS OF SKIN GLAND CELLS IN BOXFISH BEFORE AND AFTER STRESS SECRETIONS

<table>
<thead>
<tr>
<th></th>
<th>Total Mucous Cells</th>
<th>Club Cells</th>
<th>Total Club Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage II-III</td>
<td>Stage IV-V</td>
<td>Empty</td>
</tr>
<tr>
<td><strong>Before stress</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of total</td>
<td>5.5</td>
<td>11.0</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>(31)</td>
<td>(15)</td>
<td>(54)</td>
</tr>
<tr>
<td><strong>After stress</strong></td>
<td>21.7</td>
<td>7.8</td>
<td>28.0</td>
</tr>
<tr>
<td>% of total</td>
<td>(16)</td>
<td>(23)</td>
<td>(58)</td>
</tr>
</tbody>
</table>

1 Based on 6 sections totalling 16.3 mm (16,300 µ).
2 Based on 6 sections totalling 14.3 mm (14,300 µ).
SPOT TESTS OF OSTRACIN

"Spot" tests have been employed by several workers (see Pearse, 1960) to correlate the staining properties of a purified substance with the staining of certain cell inclusions in order to develop a histochemical test for the desired substance in the cell. There are some objections to the approach. One is that it has been shown that the staining properties and histochemical characteristics of "secretion granules" in gland cells often bear no relationship to the chemical and physiological properties of the products secreted by the glands. "Serous" granules are a good example of this as they often show no similarities in staining and histochemical properties with the actual secretory product of the serous glands (Gabe and Arvy, 1961).

Nevertheless, there are types of glands in which the "secretion granules" retain their staining properties, histochemical characteristics and their shape after extrusion. Mucous glands of vertebrates and the ink sac gland of Sepia are examples (Gabe and Arvy, 1961).

Thus, "spot tests" were employed to find out whether relationships between the staining properties of the assumed glandular secretory products and crude and semi-pure ostracin exist.

Materials and Methods

Drops of crude and semi-pure ostracin were placed on microscopical slides followed by a few drops of Bouin's
fluid. The slide was heated gently until all water had evaporated and was set in 70% ethanol to remove the picric acid. The slides were then processed like paraffin tissue sections and mounted in balsam.

**Results**

The addition of Bouin's fluid to a clear aqueous solution of semi-pure ostracin caused formation of a colloidal precipitate (ppt.) stained yellow by picric acid. It was noted earlier that the mucous secretions of boxfish killed in Bouin's would often congeal over the skin in a thick layer. This mucoid covering was mounted on a slide, stained with Mallory's triple and was found to consist of a mat of globules which resembled the colloidal secretory products of club cells stages IV and V (see Figure 42).

**Mallory's Triple Stain**

This discovery led to the Bouin's fixation and Mallory's triple staining of the toxic supernatant fluid and non-toxic ppt. of a crude ostracin solution. The toxic supernatant residue stained predominately blue to violet although some red-orange and orange colloidal particles were present. The non-toxic ppt. stained orange to red with only sparse blue particles.

This test was repeated using semi-pure ostracin and the residue again stained predominately blue with very few red particles.
Figure 42. The mucoid coating peeled from the skin of a boxfish killed in Bouin's fluid. Note the homogenous secretions of the club cells at the surface of the coating. Mallory's triple stain. X400.
Gomori's Aldehyde Fuchsin-Trichrome Stain

The supernatant residue of a crude ostracin solution stained with these dyes was found to consist of numerous purple granules similar to those found in the mucous cells and stage II and III club cells. Green colored particles were also seen. The ppt. contained vari-colored particles, including purple granules, but these aldehyde-fuchsin positive granules were not as numerous as they were in the supernatant residue.

Staining semi-pure ostracin residues gave similar results.

PAS Method

PAS positive particles were found in the supernatant residue of a crude ostracin solution whereas such particles were absent in the precipitate. These results were confirmed with semi-pure ostracin.

PAS + Toluidine Blue

Toluidine blue, used as a counterstain after PAS staining, completely masked the PAS positive components in a supernatant residue preparation. This is in contrast to what happened in tissue sections since, after PAS treatment, the club cell secretions would not stain with toluidine blue.

All of the above tests were performed on crude and semi-pure ostracin solutions that were not completely
deproteinized. When purer extracts of ostracin were used, such as after hot acetone or chloroform extraction, results were somewhat different.

**Mallory's Triple Stain**

Acetone extracts of ostracin would not stain in these dyes, but a branching crystalline structure was formed. These crystals were not an artifact of the stain since they were found on unstained, processed slides.

**Gomori's Aldehyde Fuchsin and Trichrome Stain**

Acetone-ostracin residues contained very fine, weakly stained purple granules and vari-colored colloidal particles. No crystals were found.

**Toluidine Blue**

Toluidine blue stained acetone and chloroform ostracin residues both ortho- and metachromatically (β) but the aqua color observed in tissue sections was not seen. In the chloroform residue preparation, numerous blue, needle-like crystals were formed.

To determine what effect the fixative had on ostracin in vitro, an aqueous, semi-pure ostracin solution was mixed with 5 times its volume of Bouin's fluid, immediately forming a precipitate. The whole mixture was dialyzed for 6 hours to remove the fixative and then assayed for activity. Although the solution was still weakly ichthyotoxic after dialysis and foamed when agitated, it lost its toxicity when
it was heated gently to dryness and dissolved in citrate saline. It was non-hemolytic to tuna erythrocytes. The initial weak toxic reaction after dialysis was probably due to residual fixative contaminants. When picric acid was used instead of Bouin’s fluid and dialysis was carried out overnight, ostracin was completely detoxified.

At first, the "spot tests" of impure ostracin showed notable parallels with the "secretory granules" in the mucous and labial cells and presented good evidence supporting these cells as the source of ostracin. However, as purer samples of ostracin were tested, the staining properties of the test residue were considerably reduced, indicating that the "spot tests" were actually demonstrating an impurity rather than ostracin. Furthermore, it was difficult to control the amount of residue on the slide because processing caused loss of incalculable amounts of the original "spot" and it was impossible to be certain that the ostracin fraction was not lost.

**DISCUSSION**

The evidence so far presented on the cellular source of ostracin in glandular tissue has been both inadequate and contradictory. Evidently there is a complexity of factors operating within the secretory cycle which cannot be detected by simple means. The club cell "secretory products" stain variably in triple stains suggesting that chemical transformations are occurring and it is uncertain
if the coarse granules in stage III are extruded with the colloidal apical product. The inactive extracts of skin sections present a formidable, but highly intriguing, problem and the labial glands further confuse the picture.

Therefore, on the basis of the histological structure of the glands, the extraction and collection of their secretions, the staining and histochemical properties of their secretory products, the correlation of ostracin secretion with numbers of gland cells in captive fish, and parallelisms and differences of the staining properties of ostracin with the "secretory particles" within the cells, the following hypotheses are proposed:

(1) The club cells are responsible for ostracin secretion in the skin.

(2) The mucous cells, containing muco- and/or glycoproteins, are true mucous cells having no other specialized function.

(3) The labial cells contain an ostracin-like toxin but have a different function than do the skin club cells.

There are, of course, other possible hypotheses and the above proposals must not be misconstrued as final conclusions. They are only believed to be the most likely explanations of a complex, unresolved problem.
CHAPTER 18
COMPARATIVE HISTOLOGY OF THE SKIN OF COWFISH
AND OTHER PLECTOGNATHS

The correlation of a poisonous skin secretion with the presence of club cells in the skin of boxfish led to a preliminary histological study of the skin of the cowfish, Lactoria fornasini, another Hawaiian trunkfish known to secrete a saponin-like toxin. The integument of a few species of puffers and one filefish were examined for comparative purposes since the puffers were also shown to contain toxic components in their mucous secretions.

Both skin and lips of the cowfish and the skin only of four species of puffer and one filefish were fixed in Bouin's fluid and processed in the manner described for boxfish tissues (Chapter 14).

EPIDERMIS OF THE COWFISH

The structure of the epidermis of cowfish has much in common with that of boxfish. The upper layers contain numerous mucous cells (12 to 16 μ) and a thin layer of serrated squamous epithelium. The middle and basal layers contain the large (40 to 80 μ) glandular "club cells" characteristic of boxfish epidermis. Many of these cells were found in the process of extrusion of secretions through pseudo-ducts while in the middle layers of the epidermis, although no glandular "pockets" bordered by dermal ridges
were observed in the sections examined. Developmental stages containing granular and agranular "secretory products" were present in most sections.

The chief structural differences noted in cowfish epidermis are: (1) the height of the epidermal layer is greater than that of boxfish, (2) a greater number of mucous cells is found in the upper layers (usually there are 2 - 3 layers of mucous cells in comparison to one in boxfish), and (3) clear vacuoles are often found in the homogenous material in the club cells of cowfish but are not commonly seen in those of boxfish.

Figure 43 shows a typical cross section of the caudal skin of a cowfish.

**Staining Properties of the Mucous and Club Cells of Cowfish**

Using the same stains and staining procedures employed successfully on boxfish epidermis, an excellent correlation in the staining properties of the mucous and club cells of cowfish and boxfish was demonstrated. In every instance the staining reactions listed in Table XI for boxfish gland cells were identical for their counterparts in the cowfish. See Figures 44 to 47 for color photomicrographs of skin sections prepared with 4 diagnostic staining procedures.

**THE LABIAL GLANDS OF COWFISH**

Labial villi containing gland cells similar to those
Figure 43. Caudal skin of the cowfish *L. fornasini*. Its superficial resemblance to boxfish skin is apparent. Mucous and club cells (stages III - V) may be seen. Heidenhain's iron hematoxylin-triosin. X400.
Figure 44. Cowfish skin stained with Mallory's triple stain. Note the similarities in staining properties of the gland cell secretions to those of boxfish. X400.

Figure 45. Cowfish skin stained with Gomori's aldehyde fuchsin-trichrome stain. The numerous mucous cells distinguish cowfish skin from that of the boxfish (Fig. 28). X400.
Figure 46. Cowfish skin stained with the PAS reagent. Compare this photo with that of the boxfish (Fig. 28). X400.

Figure 47. Cowfish skin stained orthochromatically with toluidine blue. Identical results were obtained with boxfish skin. Note that the mucous cells are unstained. X400.
of boxfish were also found in this species. Glandular "pockets" extending into the lips and villi and dental "pockets" were also present. No extensive study was made of the labial gland system of this species, but superficially the glands resembled those of *Ostracion*.

**THE EPIDERMIS OF OTHER PLECTOGNATHS**

The skin sections of four species of puffer fish, *Canthigaster jactator*, *Arothron hispidus*, *A. meleagris* and *Diodon hystrix*, and one file fish, *Pervagor spilosoma* were fixed in Bouin's and processed as described for boxfish tissue. In some cases decalcification of the skin in 5% nitric acid was necessary.

A complete description of the epidermis of these plectognaths will not be attempted. Only the salient features will be discussed.

Mucous cells, slightly larger than those found in trunkfish skin, were the most common gland cell in the epidermis of all these species. In the puffers a few small cells with agranular inclusions were found in some sections but the club cells, so characteristic of trunkfish skin, could not be found.

However, Eger (1963) has reported gland cells with homogenous and granular inclusions around the "prickle" spines of *Arothron hispidus* and he has extracted a potent "exotoxin" from the mucous secretions and skin of this species.
These gland cells became apparent when sections through the spines of *A. hispidus* were processed. Possibly the apparent absence of these cells in the other puffers was because sections through these dermal spines were not obtained.

The staining properties of both the mucous cells of all species and the gland cells around the dermal spines of *A. hispidus* (see Eger, 1963) correlated perfectly with the staining properties of the mucous and club cells of the trunkfish. However, certain structural differences were evident in puffer epidermis: (1) the mucous cells are somewhat larger and are goblet-shaped, (2) the dermal spine gland cells resemble stage V boxfish club cells, but counterparts of stages I - IV were not found, and (3) extrusion of secretions in the middle layer of the epidermis, supposedly characteristic of true "club cells", was not demonstrated.

The epidermis of the filefish, a species with non-toxic skin secretions, consisted essentially of unspecialized epidermal cells with a few mucous cells.

**DISCUSSION**

The occurrence in the trunkfishes and puffers of glandular epidermal cells possessing identical staining properties correlates with the toxic stress secretions produced by these fish. Although the trunkfish toxins appear to be chemically quite distinct from the toxin found
in the mucous secretions of the puffers, the remarkable similarity in the staining properties of their gland cells suggest that these cells are homologous in structure.

In the venomous fishes large epidermal gland cells containing both granular and agranular "secretory products" are found to be associated with spines (see for example Halstead et al., 1955a, 1955b and 1956; Evans, 1924) and are believed to secrete venom. Habel (1931) states that such venomous glands consist of club cells which are responsible for the production and secretion of venom, and list the families of teleosts in which club cells have been found. Among these families are presumably non-poisonous fishes like the cyprinids, eels and cods. Apparently, club cells have various functions in different fishes but are primarily of a protective nature.

It was pointed out previously that the "secretory granules" of gland cells are not necessarily synonymous to the active secretory product of the gland, i.e., sometimes only the supporting substance (inactive portion) is identified in histological preparations. Thus, assuming that both trunkfish and puffer toxins are being secreted by homologous epidermal gland cells, the similarities in the staining properties of the respective cells suggest that the supporting substances, and not the toxins, are being demonstrated histologically.
Therefore, the correlation of club cells in the boxfish and cowfish with toxic skin secretions is reinforced by similar correlations in the venomous fishes, and by parallels with an "homologous" gland cell and toxic skin secretions in the puffer, A. hispidus.
The two most significant findings of this investigation are the tentative characterization of the toxic component of the stress secretions of Ostracion lentiginosus as a steroid saponin (ostracin), and the discovery of specialized skin and labial glands believed to be associated with the production and secretion of ostracin. Thus, this thesis can be divided into two major portions: the nature of the toxin of Ostracion and the histology of the poison glands. The development of a bioassay to detect ostracin aided in achieving the former and histological and histochemical studies supported the latter. The major unresolved problems are the mechanism of ostracin secretion and the precise roles of the skin and labial glands.

OSTRACIN, THE STEROID SAPONIN OF THE BOXFISH

The physical, chemical and biological properties of ostracin clearly indicate that this toxin is a steroid glycoside and may be classified as a saponin along with other plant and animal glycosides having such properties.

The evidence supporting the tentative identification of ostracin as a saponin was summarized in Table VIII, including a comparison of its general properties with those of the plant glycosides, digitonin and saponin (Merck).
But it is more meaningful to compare ostracin with saponins of animal origin, such as holothurin and the starfish saponin, and with the unidentified but clearly saponin-like toxins produced by red tide dinoflagellates. Table XIV lists the diagnostic characteristics of ostracin and of these other marine "saponins".

It is at once apparent that ostracin has many properties in common with these marine "sapotoxins". From the information available, all foam profusely in water, are strongly ichthyotoxic and hemolytic, non-dialyzable, thermostable (one exception), unstable in alkali, insoluble in ether and form non-toxic complexes with cholesterol. High toxicity to a variety of living systems is characteristic of these substances which are many times more potent than the plant saponins.

Aside from the similarities of ostracin to such "sapotoxins", there are notable differences. Ostracin caused agglutination of erythrocytes prior to hemolysis. Such a reaction has not been reported for other hemolysins, nor could it be demonstrated for holothurin and saponin (Merck), which indicates that ostracin has unique physiological properties not possessed by other hemolysins.

The solubility of ostracin in organic solvents was greater than the other "saponins" and its high solubility in chloroform was surprising since polar substances are usually insoluble in lipid solvents. However, solubility
<table>
<thead>
<tr>
<th>PROPERTY</th>
<th>OSTRACIN</th>
<th>HOLOTHRURIN A</th>
<th>STARFISH SAPONIN</th>
<th>GYMNOGINUM TOXIN</th>
<th>PRYMESIUS TOXIN</th>
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</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>white amorphous hygroscopic powder (impure)</td>
<td>needle-like crystals (pure)</td>
<td>brown powder (pure)</td>
<td>...</td>
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<tr>
<td>Foaming ability in water</td>
<td>produces stable foams</td>
<td>produces stable foams</td>
<td>produces stable foams</td>
<td>cultures foamed</td>
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<tr>
<td>Solubility in water</td>
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<tr>
<td>water</td>
<td>v. sol.</td>
<td>sol.</td>
<td>sol.</td>
<td>v. sol.</td>
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<tr>
<td>methanol</td>
<td>sol.</td>
<td>insol. (absolute)</td>
<td>sol.</td>
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<td>insol. (?)</td>
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<td>chloroform</td>
<td>v. sol.</td>
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<tr>
<td>Heat stability</td>
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<tr>
<td>Acid-Base stability</td>
<td>acid-stable</td>
<td>acid-unstable (heat)</td>
<td>acid-unstable</td>
<td>acid-unstable</td>
<td>acid-stable</td>
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<tr>
<td>stability</td>
<td>base-unstable</td>
<td>base-unstable</td>
<td>base-unstable</td>
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<tr>
<td>Toxicity to fish</td>
<td>v. ichthyotoxic</td>
<td>ichthyotoxic</td>
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<td>ichthyotoxic</td>
<td>ichthyotoxic</td>
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<tr>
<td>Tox. to mammals</td>
<td>mod. toxic (mice)</td>
<td>v. toxic (mice)</td>
<td>toxic</td>
<td>v. toxic (mice)</td>
<td>...</td>
</tr>
<tr>
<td>Effect on RBC</td>
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<td>?</td>
<td>hemolytic</td>
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<tr>
<td>Reaction with cholesterol</td>
<td>detoxified by cholesterol</td>
<td>forms complex</td>
<td>...</td>
<td>detoxified by cholesterol</td>
<td>...</td>
</tr>
</tbody>
</table>

Sources of data: Holothurin A (Migrelli and Jakowska 1960; Yasunouchi 1955); Starfish saponin (Hashimoto and Yasumoto 1960); Gymnodinium toxin (Abbott and Ballantine 1957); Prynesium toxin (Shilo and Rosenberg 1960).
may be a reflection of the impurity of ostracin since impure substances often are more soluble than the purified compounds.

The toxicity of ostracin to mice was much less than that of the other "sapotoxins", whereas the ichthyotoxicity of partially purified ostracin compared favorably with that of purified holothurin A. Ostracin also caused, at concentrations of less than 10 ppm, 100% hemolysis of a rabbit erythrocyte suspension, whereas 100 ppm of holothurin A was necessary for complete hemolysis of rabbit erythrocytes (Nigrelli and Jakowska, 1960).

The antimetabolic effects shown by holothurin A in planaria and sea urchin plutei were not demonstrated with ostracin although a general toxic action was evident and inhibition of development occurred in sea urchin embryos.

Therefore, ostracin can be classified with these "sapotoxins" because its potency and mode of action appear to compare favorably with the echinoderm and red tide toxins, but the differences noted suggest that ostracin is distinct chemically.

The saponins, like the alkaloids, have until recently, only been isolated from plants. Within the past ten years saponins have been found in the sea cucumbers and starfish (Nigrelli, 1952; Yamonouchi, 1955; Hashimoto and Yasumoto, 1960). The isolation of ostracin from the stress secretions of the boxfish is the first time that steroid saponins have been
found in a vertebrate. The steroid genins found in the venom of the parotid glands of toads are structurally related to the sapogenins and the cardiac aglycones but these toad poisons are not glycosides. The occurrence of saponins in the Animal Kingdom, and especially in the Vertebrata, could be of great significance in tracing the paths of phylogeny by the biochemical evolution of the steroids and their glycosides.

THE SIGNIFICANCE OF THE SKIN AND LABIAL GLANDS TO OSTRACIN SECRETION

The discovery of histologically distinct glands in the integument and oral cavity of *O. lentiginosus*, both of which correlate with ostracin secretion, complicates the problem of precisely identifying the actual source and site of ostracin secretion. For simplicity's sake, ostracin has been considered as one toxin and there is, as yet, no concrete contrary evidence. Nevertheless, two or more saponins are often found naturally occurring together in the same species. Two distinct saponins have been isolated from the sea cucumber (holothurin A and B) and also from the starfish. Similar examples may be found in the plant saponins. In such cases, usually only one saponin is highly potent thus masking the presence of the other components in a crude extract bioassay. Therefore, it is reasonable to suspect that "ostracin" may consist of different components as does holothurin. The difficulties met in obtaining
adequate samples of ostracin from the labial glands and the failure to successfully prepare paper chromatograms showing separation of different toxic components has militated against this hypothesis, although this possibility has definitely not been disproved and should be borne in mind while considering which gland cells are responsible for "ostracin" secretion.

It has been established that two distinct gland cells, the so-called "mucous" and "club cells" occur in the integument, whereas only one type, the labial cells, form the labial glands in the buccal cavity. Therefore, several theories as to the source of ostracin are possible. In the integument either one or both gland cells may secrete ostracin, while the labial cells are the sole suspects in the labial villi glands in the oral cavity, although the gland cells of the lip epidermis may contribute to the oral secretions.

There appears to be little doubt that the skin stress secretions are the major source of ostracin since severing the lips from freshly captured boxfish did not seriously affect the total yield of ostracin from such fish. Histological and histochemical studies of the skin showed that both gland cells are actually "mucous secreting" cells. One is a common type found in the epidermis of many fishes and was simply called a "mucous" cell. Its mucins are muco- and/or glycoproteins. The second type, the so-called
"club cells", appears to be a specialized secretory cell that has been reported to occur in several venomous fishes and in a few families of non-poisonous teleosts. These "club cells" are believed to be modified serous cells (Rabl, 1931) but according to Gabe and Arvey (1961), the present classification of mucous and serous cells is an oversimplification and its use is questionable.

The "mucins" of the club cells are not muco- or glycoproteins but possibly mucopolysaccharides. Unfortunately it is difficult to identify polysaccharides in tissues since there are no specific tests for these substances (Hale, 1957). The homogenous secretions of club cells are orthochromatically stained by toluidine blue but stained red by mucicarmine indicating their mucoid nature. Furthermore, their staining properties clearly indicate their basophilic nature, whereas the mucous cell secretions are acidophilic. Although PAS negative, the coarse granules of restitutional stages II and III of the club cells are stained by Gomori's aldehyde fuchsin. Halmi and Davies (1953), after comparing the staining reactions of the Shiff reagent (PAS), aldehyde fuchsin and metachromasia by toluidine blue on various tissues, concluded that most metachromatic and orthochromatic PAS positive substances are also aldehyde fuchsin positive but there are some exceptions with a few PAS negative elements being aldehyde fuchsin positive. Thus, the histochemical techniques were largely unsuccessful
in elucidating the nature of the substance in the club cells, but this was expected since the recent discovery of steroid saponins in animal tissues precluded specific histochemical tests for these substances.

Fixation and staining of whole mucous secretions showed that the club cell mucins were the major component in the secretions and the numbers and relative sizes of mucous and club cells in the epidermis corroborate this observation. The non-toxic precipitate in a freshly collected crude ostracin solution consisted largely of material from the club cells and the spot tests of ostracin indicated that the impurities or supporting substances were reacting with the stains. Therefore, since the yield of ostracin from a single fish's secretions is so high (50 to 100 mg), it seems more reasonable to suspect the larger and more numerous club cells as being the source of ostracin rather than the mucous cells.

The formation of club cell "glands" in certain areas of the epidermis lends further support to the club cell theory of ostracin secretion since it seems unlikely that ordinary mucous cells would have formed so specialized a structure. Similar "club cell type" glands around the base of the spines of venomous fishes have been clearly shown to be specialized poison glands (Habl, 1931; Saunders, 1960; Russell and Emery, 1960). Thus, it is logical to propose that such is the case in Ostracion and possibly Lactoria.
The prominent labial glands in the mouth of trunkfish present contradictory evidence, suggesting that the labial gland cells and the small mucous cells in the skin are the source of ostracin. It appears that the labial glands consist of modified skin mucous cells judging by similarities in staining properties and structure. Since there are indications that the labial glands contain a hemolytic ichthyotoxin, it is reasonable to suspect that the skin mucous cells do also. However, if true, one would expect these cells to be more numerous in the epidermis than they are. Furthermore, the labial cells are distinct from the mucous cells in that they were never observed in the process of extrusion whereas the mucous cells were often seen extruding their products.

Although the oral secretions were ichthyotoxic and hemolytic, they probably accounted for less than 10% of the total toxicity of the stress secretions, and since the labial glands in the oral cavity are histologically distinct from the skin glands, it is likely that their secretory products are distinct also.

Comparative studies of other plectognaths add support to the club cell theory of toxin secretion. The epidermis of the cowfish contains club cells that resemble those of the boxfish in structure and staining properties, but although the toxic secretions of this species are similar in action to ostracin, they appear to be much less toxic.
The mucous cells in the superficial layer of the epidermis were more numerous in the cowfish, thereby providing a negative correlation with toxicity.

"Homologous" gland cells are found in the epidermis of puffers, which possess toxic skin secretions, and although the chemical nature of their toxins is different from ostracin, the staining properties of the secretory materials within the gland cells correlate perfectly with those of the trunkfish.

The failure to extract ostracin from the suspected poison glands in the boxfish was at first puzzling and disturbing. However, it was shown later that ostracin is highly toxic to the boxfish and causes both agglutination and hemolysis of boxfish erythrocytes in vitro. Obviously, the boxfish must have some control over its own potent toxin and since ostracin is not a protein, immunological protection is unlikely. Therefore, either ostracin is stored in glands which erect an ostracin-impermeable barrier to the body fluids or an inactive precursor accumulates in the glandular tissue and is activated by an enzyme or other means during extrusion. The latter explanation appears more likely in view of the inability to extract ostracin from the poison glands.
The main objectives of this thesis were to characterize chemically the ichthyotoxin secreted by distressed boxfish Ostracion lentiginosus and to describe the histology of their poison glands. The major results of these investigations are:

(1) An ichthyotoxic substance was found in the foamy mucous secretions of distressed boxfish.

(2) This toxin was isolated and partially purified by filtration, centrifugation, boiling and dialysis of the mucous secretions and extraction of the water soluble residue with acetone or chloroform. The toxic extract so prepared was named "ostracin".

(3) Ostracin was shown to be toxic to a variety of aquatic organisms and, in general, it exhibited the same effect on living systems as does holothurin (the sea cucumber toxin), the starfish saponin and the red tide toxins of Gymnodinium veneficum and Pyrnesium parvum.

(4) Ostracin was only moderately toxic to white mice. About 4 mg of the purest preparation injected intraperitoneally was needed to kill a 20 g mouse, whereas this same preparation, diluted as great as 1:1,000,000 was lethal to fish.

(5) Since ostracin was toxic to most marine fishes at concentrations as low as 1 ppm, a fish immersion bioassay
was developed to detect ostracin in crude and semi-purified preparations.

(6) Ostracin was detected in the stress secretions of boxfish collected, regardless of maturity, sex, time of the year caught or habitat, suggesting that ostracin is an endogenous toxin.

(7) Ostracin is thermostable, non-dialyzable, stable in acid and unstable in alkali at room temperatures, but is readily hydrolyzed by heating in strong acid.

(8) Ostracin is soluble in water, methanol, ethanol, acetone and chloroform but is insoluble in diethyl ether and benzene.

(9) Profuse foaming in aqueous solutions was characteristic of all ostracin preparations.

(10) Ostracin was shown to be hemolytic to vertebrate erythrocytes and to form a water-insoluble, non-toxic complex with cholesterol. Thus, ostracin was tentatively identified as a steroid saponin.

(11) Besides its hemolytic activity, ostracin was found to cause agglutination of erythrocytes of some species, a property which distinguishes it from other saponins.

(12) The boxfish is poisoned by its own toxin, however it is more resistant to ostracin than other fish. Both hemolysis and agglutination of boxfish erythrocytes by ostracin occurred in vitro.

(13) The cowfish, Lactoria fornasini and two other species
of Hawaiian trunkfishes give off an ichthyotoxic, hemolytic stress substance with their foamy mucous secretions.

(14) Several species of puffer-fishes also release a toxin with their mucous secretions. However, the puffer toxin is different from the trunkfish toxin and appears similar in action to Tetraodotoxin.

(15) Ostracin was readily detected both in the oral exudations and the skin mucous secretions of boxfish but could not be extracted from the corresponding tissues of freshly killed fish, suggesting that ostracin is activated only during extrusion by the glands.

(16) A histological investigation of the skin (Bouin's fixation) showed that 3 cell types occurred in the epidermis: ordinary, unspecialized epidermal cells, mucous cells and specialized gland cells called club cells.

(17) Clusters of club cells glands were found in dermal pockets on some areas of the skin, especially in the carapace epidermis behind the pectoral fin.

(18) Suggested developmental stages of club cells were described from study of Bouin's-fixed, Heidenhain's iron hematoxylin sections.

(19) Some staining properties of the "secretory" material within the mucous and club cells were determined with Harris hematoxylin-triosin, Heidenhain's iron hematoxylin, Mallory's triple stain, Gomori's aldehyde fuchsin-trichrome stain, Toluidine blue, Periodic acid-Achiff's (PAS),
Dresbach's mucicarmine and Sudan Black B.

(20) The fine granules in the mucous cells and the homogenous secretions in the club cells have distinctive staining properties. The mucous cells contain muco- and/or glycoprotein whereas the material in the club cells was not identified by histochemical techniques.

(21) Club cells were found extruding their products in the middle layers of the epidermis, supposedly characteristic of such cells. Only club cell stages III - V appeared to be capable of extrusion.

(22) Labial glands of a compound acinous type were found in the oral cavity of boxfish and are believed to be associated with the toxic oral secretions although toxic extracts of these glands could not be consistently obtained.

(23) The labial gland system consisted of dorsal and ventral labial villi and dermal glandular pockets extending into the lips and emptying both inside and outside the oral cavity.

(24) Only one type of secretory cell was recognized in the labial glands. It resembled the mucous cells of the skin in structure and staining properties but appeared to be a distinct gland cell derived from the epidermis of the lips.

(25) Both the skin and labial glands of starved boxfish held in captivity were characterized by a great proliferation of epidermal cells and reduction in numbers of gland cells. This correlated with a significant decrease in the ostracin
secretion of such fish.

(26) "Spot tests" of ostracin and a controlled stress experiment were inconclusive and did not aid in localizing ostracin within the gland cells.

(27) Histological comparison of the skin of the cowfish Lactoria fornasini with that of the boxfish showed excellent correlations with the structure and staining properties of the mucous and club cells of this species. Similar results were obtained with the skin of some puffers, whereas the skin of a non-toxic filefish had no specialized gland cells.

(28) It was proposed, based on the histological and experimental evidence, that the club cells in the epidermis of boxfish are the major source of ostracin secretion, but the labial cells, and possibly the mucous cells of the skin, may also be associated with the stress secretion.


Brock, V. E. 1955. Possible production of a substance poisonous to other fish by the boxfish Ostracion lentiginosus Schneider. Copeia. 3 (195-196).


LITERATURE CITED (continued)


LITERATURE CITED (continued)


LITERATURE CITED (continued)

Rosen, N. 1912a. Studies on the plectognaths. The blood-

———. 1912b. Studies on the plectognaths. The air-
sac with notes on other parts of the intestines.

———. 1913a. Studies on the plectognaths. The

———. 1913b. Studies on the plectognaths. The body

Ruggieri, G. D. and R. F. Nigrelli. 1960. The effects of
holothurin, a steroid saponin from the sea cucumber, on


Proc. of Second International Congress of Surface Activity.
IV: 271-276.

Saunders, P. R. 1960. Pharmacological and chemical studies
of the venom of the stonefish (genus Synanceja) and

Schultz, L. The fishes of the Marshall Islands and Marianas. III.
(Manuscript).

Schantz, E. J. 1960. Biochemical studies on paralytic

principles formed by the chrysomonad Prymnesium parvum

Alarm reaction of the top smelt, Atherinops affinis (Ayres).
Science. 138: 681-682.

Sokolskaya, A. M. 1951. The saponin of the roots of
Patrinia intermedia R. et Schult. Soviet Pharmaceutical
Research. III: 378-380. Pharmacognosy. (Translated into
English by Consultants Bureau Inc.)
LITERATURE CITED (continued)


