exponential growth. However, the conversion under these conditions is neither as rapid nor as synchronous as it is under the conditions already described. Furthermore, although glycerol gives optimum results, 0.012*M* phenethyl alcohol, under the same conditions, also induces formation of microcysts.

While the refractile spheres produced by the glycerol technique are microcysts from a morphological point of view and are capable of almost 100 percent germination efficiency, other parameters indicate that, physiologically, they are not mature microcysts for at least an additional 12 hours; for example, the ability to respire on Casitone does not disappear until about 12 hours have passed.

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References and Notes

1. M. Dworkin and H. Voelz, *J. Gen. Microbiol.* **28**, 81 (1962).
2. J. Adye and D. Powelson, *J. Bacteriol.* **81**, 780 (1961).
3. J. Mager, M. Kuczynski, G. Schatzberg, Y. Avi-Dor, *J. Gen. Microbiol.* **14**, 69 (1956).
4. M. Dworkin, *J. Bacteriol.* **84**, 250 (1962).
5. Supported by NSF grant GB-9 of the Developmental Biology Program and a PHS career development award (I-K3-GM-5869-10) to M.D.

17 July 1964

Ostracitoxin: An Ichthyotoxic Stress Secretion of the Boxfish, Ostracion lentiginosus

Abstract. *Boxfish under stress produce an ichthyotoxic, hemolytic, nonprotein poison in the mucous secretions of their skin. This heat-stable, nondialyzable "ostracitoxin" foams profusely in aqueous solutions and is toxic to various biological systems. It is apparently unique among known fish poisons; it is toxic to boxfish and resembles red tide and sea cucumber toxins in general properties.*

The boxfish, *Ostracion lentiginosus*, is a member of the trunkfish family (Ostraciontidae), a group of tropical marine teleosts characterized by a rigid dermal carapace encasing the body. Trunkfishes are often confused with their close relatives, the puffer fishes (*1*), but, whereas the poisonous nature of puffers is well known (*2*), information on the toxic nature of trunkfishes is sketchy.

It has been suspected that trunkfishes under stress exude a substance poisonous to other fishes (*3, 4*). Introduction of newly captured, highly excited trunkfish into aquaria with other fishes often results in the death of all other fish inhabitants within minutes. Such rapid mortality cannot be attributed to anoxia, and it has been postulated that a poisonous substance is produced by distressed trunkfish.

Boxfish out of water secreted a copious, watery mucus which foamed profusely on agitation. The toxic nature of this mucus was convincingly demonstrated by rinsing skin secretions into the aquarium water of other reef fishes. Symptoms exhibited by fish so exposed were initial irritability and gasping, followed by quiescence characterized by decrease in the rate of opercular movements; loss of equilibrium and locomotion and sporadic convulsions and death. No recovery occurred after the initial symptoms appeared.

Mucous secretions of the skin of distressed boxfish were collected by placing newly captured, living boxfish into small containers, adding 10 to 50 ml of a distilled water rinse, and swirling the fish around for five minutes. Such stress caused the boxfish to secrete a foamy mucus which was collected in the rinse water. A standard bioassay was established by adding a portion of the total rinse volume to 100 ml of sea water containing four to six newborn sailfin mollies, *Mollienesia latipinna*, 10 to 12 mm long. The mean survival time was used as an index of toxicity.

Preliminary isolation techniques (*5*) included pooling the filtered aqueous rinses of six to eight boxfish, centrifuging at 16,000 rev/min, and dialyzing against tap water. The resulting clear supernatant fluid retained its original toxicity, whereas both the precipitate and dialyzate were nontoxic. Heating the toxic supernatant fluid caused further precipitation but no reduction in toxicity. Boiling the solution to dryness caused no appreciable loss in toxicity of the residue, clearly demonstrating the nonprotein nature of the poison.

The crude toxic residue was soluble in water, methanol, ethanol, acetone, and chloroform, but insoluble in diethyl ether and benzene. It was stable in acid (*p*H 2.0) and mildly basic (*p*H 11.0) solutions, but was rapidly detoxified upon excess addition of a strong base (KOH). Before heating, the toxicity of fresh secretions gradually decreased at refrigeration temperatures, while boiled solutions could be maintained for months at these temperatures with little loss in toxicity, indicating that bacterial detoxification was occurring.

Repeated extraction of the dried residue with acetone or chloroform and diethyl ether resulted in a particulate substance which formed stable foams in aqueous solutions and was toxic to the assay fish at concentrations of 1 : 1,000,000. Approximately 50 to 100 mg of this crude toxin could be obtained at one time from the skin secretions of a single adult boxfish (*6*). The toxic principle of these secretions will be referred to as "ostracitoxin," using the generic (or familial) name of the boxfish in accordance with the naming of other toxins such as tetrodotoxin-tarichatoxin, bufotoxin, and holothurin.

Aqueous and ethanolic extracts of the skin, viscera, and muscles of freshly killed boxfish were, surprisingly, nontoxic. Ostracitoxin was detected only in the epidermal mucous secretions of living, distressed boxfish. This is in sharp contrast with tetrodotoxin, which is found in the skin and viscera of many species of puffer fish and was only recently found in large amounts in the skin secretions of a few puffers (*7, 8*). The boxfish paradox suggests that ostracitoxin is "activated" during secretion. The susceptibility of the boxfish to its own toxin also supports this hypothesis: intramuscular injections of fresh mucous secretions caused almost immediate loss of balance, and death occurred within a few minutes. And although boxfish are much more resistant to ostracitoxin in solution than are other fishes, they become moribund when left in sea water with a high concentration of ostracitoxin (*9*). Puffer fishes, on the contrary, are immune to tetrodotoxin.

A survey of the effects of ostracitoxin solutions on various biological systems indicated that ostracitoxin has high biologic activity. For example, ostracitoxin caused desensitization of sea anenome and hydroid tentacles, inhibit-

ed the cleavage of sea urchin blastomeres, and was generally toxic to many invertebrates. Fishes were highly susceptible to ostracitoxin, and all but a few (*10*) were rapidly killed by immersion in sea water containing this toxin.

Intraperitoneal injections of ostracitoxin into white mice quickly caused ataxia, labored breathing, coma, and death. However, the minimum lethal dose of crude ostracitoxin to mice was quite high (0.2 mg/g of mouse) precluding the use of mice for bioassay. Sublethal injections into mice caused symptoms of poisoning, but recovery was complete, unlike ostracitoxin's irreversible action on fish.

Ostracitoxin caused hemolysis of vertebrate erythrocytes in vitro at concentrations as low as 1.0 part per million. A marked agglutination reaction preceded hemolysis when ostracitoxin was added to fish erythrocytes in citrated or heparinized saline; such agglutination was not observed with human or mouse blood, but rabbit erythrocytes were agglutinated as readily as those of fish. The minimum effective concentration for agglutination of fish erythrocytes of the skipjack tuna (*Katsuwonus pelamis*) was 1 : 20,000. Agglutination in vitro also preceded hemolysis in boxfish erythrocytes, providing further evidence of the toxicity of ostracitoxin to boxfish.

Ostracitoxin is clearly unique among all known fish toxins. Its ichthyotoxic property alone—that is, its property of poisoning fish immersed in ostracitoxin solutions—distinguishes it from tetrodotoxin and ciguatera toxin. In this respect ostracitoxin closely resembles certain red tide, sea cucumber, and starfish toxins (*11*). The many similarities between ostracitoxin and holothurin A, and the clearly saponin-like properties (*12*) of crude ostracitoxin, indicated that the boxfish toxin might be a steroid saponin (*7*). However, preliminary studies now in progress with the pure toxin indicate that ostracitoxin is not a saponin (*13*).

Although ostracitoxin has been identified only in the stress secretions of the boxfish, *O. lentiginosus*, preliminary studies showed that the mucous secretions of three other Hawaiian trunkfishes, *Lactoria fornasini, L. diaphanus*, and *Rhynchostracion* sp. were ichthyotoxic and hemolytic. Furthermore, a Red Sea boxfish (*2*) and an Atlantic trunkfish (*4*) have already been reported to be poisonous to fish. It appears that ostracitoxin-like poisons may be as characteristic of the Ostraciontidae as tetrodotoxin is of the Tetraodontidae and Salamandridae. Such a biogenetic finding may be of systematic importance within the trunkfish group and suborder Balistoidae.

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References and Notes

1. Trunkfishes and puffer fishes are of the order Tetraodontiformes (Plectognathi) but belong to different suborders.
2. H. S. Mosher, F. A. Fuhrman, H. D. Buchwald, H. G. Fischer, *Science* **144**, 1100 (1964).
3. E. Clark and A. F. Gohar, *Publ. Marine Biol. Stn. Al Ghardaqa* **8**, 70 (1953); R. P. L. Straughan, *The Salt Water Aquarium in the Home* (Barnes, New York, 1959), p. 213.
4. V. E. Brock, *Copeia* **3**, 195 (1955).
5. Ostracitoxin was recently purified by David Boylan, Department of Chemistry, University of Hawaii.
6. Adult boxfish range from 110 to 200 mm in total length.
7. D. A. Thomson, dissertation, Univ. of Hawaii (1963).
8. W. H. Eger, thesis, Univ. of Hawaii (1963).
9. Assay concentrations between 10 and 100 parts per million, fatal to reef fishes within 10 minutes, had no effect on boxfish. The minimum lethal dose to boxfish could not be readily determined because distressed boxfish continually added toxic secretions to their aquarium water.
10. Besides the boxfish, the pearl fish, *Carapus homei*, an inquiline in the cloaca of certain sea cucumbers and starfishes, was as resistant to ostracitoxin as it was to crude holothurin, the steroid saponin of the sea cucumber.
11. B. C. Abbott and D. Ballantine, *J. Marine Biol. Assoc. U.K.* **36**, 169 (1957); M. Shilo and R. F. Rosenberger, *Ann. N.Y. Acad. Sci.* **90**, 866 (196➡ J. A. Gates and W. B. Wilson, *Limnol. Oceanog.* **5**, 171 (1960); R. F. Nigrelli and S. Jakowska, *Ann. N.Y. Acad. Sci.* **90**, 884 (1960); Y. Hashimoto and T. Yasumoto, *Nippon Suisan Gakkaishi* **26**, 1132 (1960).
12. Like the saponins, crude ostracitoxin is toxic to fish and aquatic organisms and to living systems in general; it is heat stable, detoxified by cholesterol, and cannot be dialyzed. It also resembles the saponins in foaming ability and solubilities.
13. D. Boyland, personal communication (1964).
14. I thank the Hawaii Marine Laboratory, University of Hawaii, and the Honolulu Biological Laboratory, U.S. Bureau of Commercial Fisheries, for use of their facilities. This research was part of a doctoral dissertation and was supported in part by a grant from the Hawaii Marine Laboratory. This is contribution No. 209, Hawaii Marine Laboratory, University of Hawaii.

11 August 1964

Tuberculin Reactivity of a Carbohydrate Component of Unheated BCG Culture Filtrate

Abstract. *Carbohydrate and protein fractions from the filtrate of a culture of* Mycobacterium bovis *strain BCG have equal activity in sensitive guinea pigs. The sequential action of two proteolytic enzymes caused little alteration in the reactivity of the carbohydrate but almost completely eliminated the reactivity of the protein.*

Carbohydrate components of mycobacterial culture filtrates have usually been considered incapable of eliciting a tuberculin reaction in sensitive animals (*1*). Our previous study (*2*), however, indicated that a carbohydrate fraction, GA, isolated from unheated filtrates of cultures of *Mycobacterium bovis* strain BCG may induce a tuberculin reaction indistinguishable from that induced by protein fractions. It also has been reported recently that a lipopolysaccharide derived from *M. tuberculosis* may have tuberculin activity (*3*).

Because the carbohydrate fraction, GA, contained approximately 10 percent protein, a question arose over the role of protein in the tuberculin activity of the carbohydrate. This report describes the results of proteolytic digestion of fraction GA and establishes that the carbohydrate is, in fact, capable of inducing a tuberculin reaction in sensitive guinea pigs indistinguishable in intensity, appearance, and time of development from that induced by a protein fraction FB or by PPD-S (*4*).

Solutions of FB and GA (25 mg/ml) were prepared in 0.03*M* phosphate buffer, *p*H 7.5, and ethanol was added to a concentration of 4 percent. To 2.0 ml portions of each of these solutions 0.8 mg of Pronase, a prote-

Table 1. Yields and nitrogen analyses of enzyme and nonenzyme treated components isolated by Sephadex G-25 chromatography.

Substance	Yield* (mg)	Nitrogen (%)
FBS	27.8	10.4
FBES	6.3	6.8
GAS	22.2	1.1
GAES	32.9	0.4

* Yield is based on only that portion of the eluate that was salt-free.