Toxins from Eggs of Fishes and Amphibia

Frederick A. Fuhrman, Geraldine J. Fuhrman, David L. Dull, and Harry S. Mosher

The occurrence of poisonous eggs among fishes is reviewed. Authentic instances of poisoning can be attributed to eggs of fish from about 10 genera. The best known egg toxin, tetrodotoxin, from the eggs of puffer fish (order *Tetraodontiformes*) and newts (*Salamandridae*) is an aminoperhydroquinazoline compound ($C_{11}H_{17}N_3O_8$) of proven structure. It is extremely toxic (LD_{50}=10 μg. per kg.) and blocks conduction in nerves by selectively preventing the increase in conductance to sodium ions that normally accompanies excitation. The eggs of the gar contain a toxin of high molecular weight that produces gastrointestinal symptoms in animals and is definitely not tetrodotoxin. The eggs of the marbled sculpin, or cabezon (*Scorpaenichthys marmoratus*), contain a toxin that inhibits growth of cells in tissue culture and produces necrosis in the liver and spleen. The toxin appears to be a protein or is attached to protein in the eggs.

Man's predilection for caviar has caused him to search far beyond the sturgeon for equally palatable roe. The search has many times resulted in gastrointestinal distress and, occasionally, in more severe symptoms, and even in death. The literature on poisonings from eating fish roe (ichthyotoxism) has been reviewed recently by Halstead (1967). Many of the older reports are unreliable because of failure to recognize bacterial spoilage as one common cause of toxicity. Others record only one instance of poisoning from eating the roe of a given fish, and subsequent reports of toxicity of that roe are lacking. Many citations of toxicity appear to be transferred from one review to another without critical evaluation. When reports of doubtful authenticity are eliminated it is possible to list about a dozen fish whose roe can be documented to be poisonous (Table I).

The gars are large, sluggish fish found largely in fresh and brackish waters of the southeastern United States, Cuba, and Central America. The roe of at least three species is poisonous and is discussed below.

The pikes are fresh-water fish found in North America, Asia, and Europe. There are several old reports that the roe is poisonous when eaten, and these were substantiated by McCrudden (1921), who produced mild poisoning by feeding fresh and dry pike roe to cats and dogs and severe poisoning and death by intravenous injection of extracts of roe into rabbits. The toxin appeared to be a protein and was heat-labile.

There is suggestive evidence that the roe of several carps is poisonous, and McCrudden (1921) reported experimental evidence that roe of one such fish, the barbel, was poisonous in animals and that the toxin resembled that from pike roe.

Max C. Fleischmann Laboratories of the Medical Sciences and Department of Chemistry, Stanford University, Stanford, Calif. 94305

Two species of killifish, known commonly as mummichog, from Canada, have been reported to produce toxic effects but not death when fed to a variety of pet animals (White *et al.*, 1965). There is suggestive evidence that the toxin is present in the roe.

Halstead and Schall (1954) reported that intraperitoneal injection of aqueous extracts of the roe of *Paranthias furcifer*, a member of the family that includes the seabass and groupers, caught off the coast of Ecuador, was toxic in mice.

The roe of the cabezon was shown to be toxic by Hubbs and Wick (1951) and further investigation of this toxin forms a major part of this report.

The Japanese prickelback or blenny, *Stichaeus* (*Dinogunellus*) *grigorjewi* (Herzenstein), has been used for the preparation of *kamaboko* (boiled fish paste) and *soboro* (boiled seasoned and crushed fish), and the fish is sold fresh for food on the island of Hokkaido. Asano and Itoh (1962) cite two recent instances of poisoning (one fatal) in human beings after eating boiled blenny roe, and state that the poisonous nature of the roe is known generally by the people of Hokkaido. The symptoms of poisoning are diarrhea, nausea, vomiting, and a sensation of constriction in the chest. The toxin is nondialyzable, destroyed by autoclaving at 120° C. (but not by boiling), soluble in water and dilute saline, and is precipitated from these solutions during deproteinization (Asano and Itoh, 1962). Asano and Itoh prepared a "lipoprotein" from blenny roe using Chargaff's method for lipovitellin, and found this to produce death in 24 to 48 hours when injected into mice. However, lipids extracted from the roe also proved to be toxic. Both lipoprotein and lipids were reported to produce fatty infiltration in the liver of rats. Hatano *et al.* (1964) attempted to fractionate blenny roe toxin by extraction with solvents but found the toxicity to be associated with numerous lipid fractions and never to exceed about 260 mg. per kg. when

Table I. Fish Known to Have Poisonous Roe

Common Name	Scientific Name	Reference
Gars	*Lepisosteus spatula* (Lacépède)	Coker, 1930
	Lepisosteus osseus (Linnaeus)	Netsch and Witt, 1962
	Lepisosteus productus (Cope)	
Pikes	*Esox lucius* (Linnaeus)	McCrudden, 1921
Barbel	*Barbus barbus* (Linnaeus)	McCrudden, 1921
Killifishes	*Fundulus diaphanus* (LeSueur)	White *et al.*, 1965
	Fundulus heteroclitus (Linnaeus)	
Creolefish	*Paranthias furcifer* (Valenciennes)	Halstead and Schall, 1954
Cabezon	*Scorpaenichthys marmoratus* (Ayers)	Hubbs and Wick, 1951
Blenny	*Stichaeus grigorjewi* (Herzenstein)	Asano and Itoh, 1962
Puffers	*Fugu rubripes rubripes* (Temminck and Schlegel)	Halstead, 1967 for Ref.
	Fugu niphobles (Jordan and Snyder)	
	Lagocephalus sceleratus (Forster)	
	Arothron hispidus (Linnaeus)	
	Sphaeroides maculatus (Block and Schneider)	
	(And many others)	

injected intraperitoneally in mice. Subsequently, Asano (1964) isolated two different complex toxic lipids and Asano and Itoh (1966) obtained several lipoprotein fractions. Only one of these, delta-lipostichaerin, was toxic when administered orally.

Various puffer fish have poisonous roe from which tetrodotoxin has been isolated. This toxin is discussed in detail here.

TETRODOTOXIN

Various fishes belonging to the order Tetrodontiformes, commonly known as puffers, swellfish, or *fugu*, have been known for thousands of years to be poisonous when eaten. The earliest Chinese pharmacopeia, *Pen-T'so Chin* or *The Herbal*, which dates from the first or second century B.C. or earlier, included puffer eggs among its drugs (Kao, 1966). Yokoo (1950) isolated from the eggs of the puffer, *Fugu rubripes rubripes* (=*Sphoeroides rubripes*), a crystalline substance (originally named spheroidine), now known as tetrodotoxin, that is among the most toxic nonprotein compounds known. The LD_{50} in mice is about 10 μg. per kg.

At Stanford University in the early 1930's, Twitty, an embryologist, showed by grafting experiments in amphibia, that the eggs of the common California newt, *Taricha torosa*, contained a potent, diffusible, neurotoxin (Twitty, 1966). Although the pharmacological properties of the toxin were established at this time, little progress was made on its chemistry (Mosher *et al.*, 1964). By 1960, the authors thought it appropriate to renew attempts to isolate the toxin, and it was not long before Brown and Mosher (1963) obtained from eggs of the California newt (Figure 1) a crystalline toxin (tarichatoxin) having an LD_{50} in mice of about 10 μg. per kg. This toxin proved to be identical in every respect with a sample of tetrodotoxin, kindly

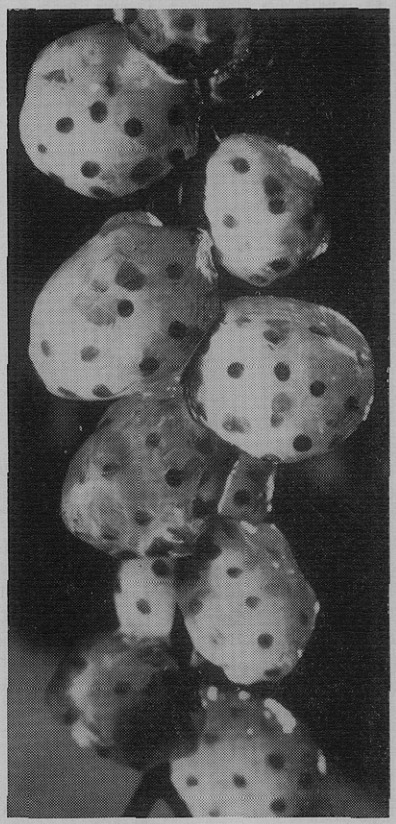

Figure 1. Clusters of eggs of the California newt, *Taricha torosa*, attached to a branch

provided by Kyosuke Tsuda, isolated from the ovaries of puffer fish.

Identity of Tetrodotoxin from Eggs of Puffers and Newts. While many of the details of this work have been presented elsewhere (Mosher *et al.*, 1964), the occurrence of the same potent toxin in the eggs of animals as diverse as puffer fish and newts is a fact of such unusual biological interest that the evidence for their identity will be summarized here. When it was found that the newly crystallized toxin from newt eggs had an LD_{50} in mice of not over 10 μg. per kg., it was immediately apparent from data on toxicity alone that if it were any known toxin it must be either tetrodotoxin or saxitoxin, as Brown and Mosher noted in their report announcing the isolation of the crystalline toxin (Brown and Mosher, 1963). [The somewhat more toxic substances from the skin of the frog, *Phyllobates* (Daly *et al.*, 1965; Märki and Witkop, 1963) and ciguatoxin (Scheuer *et al.*, 1967) had not yet been isolated.] Differences in molecular formulas and in chemical properties made it unlikely that the toxin from newts' eggs was identical with saxitoxin from shellfish. However, each new comparison of the toxin from newt eggs with the sample of tetrodotoxin from puffer ovaries failed to reveal any differences. Neither sample of toxin had an informative ultraviolet absorption spectrum, both decomposed without melting, and both were nonvolatile, so that comparison of mass spectra could not be made. The nuclear magnetic resonance (NMR) spectra were identical, but were of such a nature that small differences probably would not be detected. Toxins from both sources showed identical

behavior when compared directly by thin-layer chromatography using four different solvent systems. Further, several acetate derivatives of toxin from the two sources were identical with respect to melting point, mass spectra, NMR spectra, infrared spectra (Figure 2), and optical rotation (Buchwald *et al.*, 1964). The conclusion that newt eggs contained tetrodotoxin was inescapable on chemical grounds, but was further strengthened by pharmacological data. The median lethal doses and general pharmacological effects in mammals were identical. The newt *Taricha*, however, could withstand at least 1000 times the mouse LD_{50} of toxin from its own eggs without effect, and tetrodotoxin from puffer ovaries proved to be equally inocuous. Ishihara (1918) had found that of 22 species of animals tested, only the puffer fish itself and the Japanese newt, *Cynops pyrrhogaster* [now known to contain tetrodotoxin (Wakley *et al.*, 1966)], were resistant to puffer toxin. Moreover, the resistance was a property of the nerves themselves, and isolated nerve axons of newts and puffer fish were resistant to toxins from both sources (Kao and Fuhrman, 1967).

Narahashi *et al.* (1964) found that tetrodotoxin blocks conductance in nerve axons by a highly specific action on sodium conductance described in more detail below. The toxin from newt eggs had an identical effect (Takata *et al.*, 1965).

Thus, the inescapable fact is that the eggs of puffer fish and newts both contain tetrodotoxin. The biological significance of the occurrence of this toxin in the eggs of two very different animals is not yet evident.

Occurrence and Distribution of Tetrodotoxin. Tetrodotoxin has been isolated in crystalline form only from the ovaries and livers of several species of the genus *Fugu* (=*Sphoeroides*), from the eggs of *Taricha torosa* and *T. rivularis*, and from whole adult *T. torosa* (Wakley *et al.*, 1966). Many other tetraodontoid fish are known to be poisonous (Halstead, 1967; Kao, 1966), and all newts of the family Salamandridae so far examined contain a similar toxin (Wakley *et al.*, 1966). However, these latter toxins have not been isolated and their identity with tetrodotoxin is only probable. Conceivably, these other puffers and newts contain unidentified toxins closely related to, but not identical with, tetrodotoxin. Against this possibility is the evidence that any alteration in the molecular structure of tetrodotoxin greatly decreases its toxicity. This suggests that tetrodotoxin is unique and not a member of a series of related toxins.

In both puffer fish and newts, tetrodotoxin is found in very high concentration in the eggs and ovaries

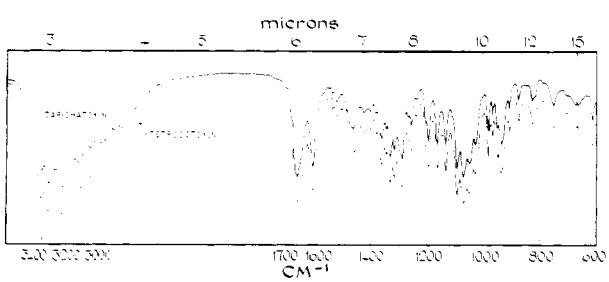

Figure 2. Infrared spectra of tetrodotoxin from newt eggs (tarichatoxin) and from puffer eggs (tetrodotoxin)

Table II. Distribution of Tetrodotoxin in Tissues of Puffer Fish and Newts

(Approximate concentration in μg. per g. fresh tissue of female animals)

	Eggs & Ovaries	Liver	Skin	Muscle
Tetrodon Fish[a]				
Fugu rubripes rubripes	100	100	1	<0.2
Fugu vermicularis porphyreus	400	200	20	1
Fugu pardalis	200	1000	100	1
Fugu vermicularis vermicularis	400	200	100	4
Fugu oscellatus obscurus	1000	40	20	<0.2
Newts[b]				
Taricha torosa	25	<0.1	25	2

[a] Data from Tani (1945) recalculated by Kao (1966) assuming 1000 Tani units = 10 μg. tetrodotoxin.
[b] Data from Wakley *et al.* (1966).

(Table II). In puffers, the toxin also occurs in high concentration in the liver and the skin, while in newts it is also present in the skin but not the liver. All puffer fish listed in Table II are important food fish in Japan or China. According to Tsuda (1966), many instances of poisoning from tetrodotoxin occur from mistaking puffer roe for the roe of other fish or from failure to remove completely the roe from the fish. The concentration of tetrodotoxin in the ovaries of puffers is highest in the winter when the ova are mature, and decreases markedly during the summer (Tani, 1945). As the ova mature and the ovaries increase in size during the fall and winter, not only does the total quantity of the toxin in the organ increase, but the eggs contain more toxin per gram. The concentration of toxin in the liver also increases at the same time. However, it is only during the winter, when the tetrodotoxin concentration of the tissues is highest, that puffers are most often eaten (Tsuda, 1966).

The only tetrodon fish common to the continental United States is the Atlantic puffer, or blowfish, *Sphaeroides maculatus* (Bloch and Schneider). The annual catch exceeds 6 million pounds (Lynch *et al.*, 1967). Lalone *et al.* (1963) reported that extracts of the ovaries (and other tissues, particularly skin) of these fish taken off Florida were frequently, but not invariably, toxic when injected into animals. The toxin was not identified but the symptoms resembled those produced by tetrodotoxin. Calculations from their data show that if the toxin is indeed tetrodotoxin the ovaries may contain about 0.06 μg. per gram. Lynch *et al.* (1967), using methods that should detect approximately that amount of toxin, could not show toxicity in any tissues of the puffers caught off New Jersey in May to July. When they extracted and concentrated ovaries using a method developed for tetrodotoxin by Johnson (1964), they obtained about 0.005 μg. per gram tissue, but large losses probably occurred. The authors have tested the toxicity of tissues of *Sphaeroides maculatus* taken off Coney Island in September. The method was the same as described for determining the toxicity of newt tissues (Wakley *et al.*, 1966). The ovaries of these fish contained a toxin that produced symptoms of tetrodotoxin poisoning and the concentration was

equivalent to about 0.05 μg. per gram tetrodotoxin. No toxicity was found in muscle and liver. Whether the differences in toxicity of tissues of *Sphaeroides maculatus* are geographical or seasonal or both is uncertain.

Pharmacology. The symptoms of tetrodotoxin poisoning may begin within minutes of ingestion and proceed with great rapidity. They consist of numbness and tingling of the lips, tongue, and mouth, muscular weakness, paralysis, hypotension, and death. The symptoms of tetrodotoxin poisoning are attributable to the effect of the toxin in blocking conduction in nerve axons. This occurs in a unique manner and has thus provided a physiological tool of great precision.

According to the Hodgkin-Huxley theory of excitation, the nerve action potential consists of a series of rapid, self-limiting changes in permeability of the nerve membrane to sodium and potassium ions following depolarization to a critical level. Tetrodotoxin selectively blocks the early transient increase in permeability of the membrane to sodium ions (Narahashi *et al.*, 1964; Takata *et al.*, 1965), leaving intact the later increase in permeability to potassium ions. Thus, the nerve fails to conduct. Other substances (*e.g.*, cocaine) block conduction by preventing changes in permeability to both ions. This highly selective action of tetrodotoxin makes it useful for elucidating those physiological processes that depend upon changes in permeability to sodium ions, although the results must be interpreted with caution (Kao and Fuhrman, 1967).

Chemistry. Tetrodotoxin is a very unusual molecule chemically as well as pharmacologically (Figure 3). It has the molecular formula $C_{11}H_{17}N_3O_8$; it thus contains one oxygen or nitrogen atom for each carbon atom. Chemical degradation of tetrodotoxin by the action of sodium hydroxide gives 2-amino-6-hydroxymethyl-8-hydroxyquinazoline (Goto *et al.*, 1962; Tsuda *et al.*, 1962; Woodward, 1964) (Figure 4), which contains all three nitrogen atoms of the tetrodotoxin molecule in a guanidine arrangement and nine of the 11 carbon atoms. However, this quinazoline derivative is aromatic, whereas tetrodotoxin is saturated and has a pK_a of 8.7 while normal guanidine derivatives have pK_a values around 12. The determination of the structure proved to be extraordinarily difficult in spite of the fact that ample supplies were available for chemical studies. The complications were associated with the difficulties encountered in obtaining the toxin in pure form, with the sensitivity of the toxin to basic as well as acidic media, and with the nonvolatility of the toxin. The latter property precluded the use of mass spectrographic methods on the toxin itself. Consequently, x-ray crystallographic analysis of derivatives played an important role in the solution of the structure and configuration of tetrodotoxin. In fact, three different groups carried out x-ray structural determinations on four different heavy atom derivatives of tetrodotoxin—namely, tetrodonic acid hydrobromide (Tsuda *et al.*, 1964); bromoanhydrotetrodic lactone hydrobromide (Goto *et al.*, 1963; Tomiie *et al.*, 1963); 6, 11-diacetylanhydrotetrodotoxin hydroiodide (Tsuda *et al.*, 1964); and 4-*O*-methyl-6,11-*O*,*O*-isopropylidinetetrodotoxin hydrobromide (Woodward, 1964). One of these studies (Tomiie *et al.*, 1964) was extended to include a Bijoet analysis, which gave the absolute configuration of the derivative. Even with these x-ray analyses on tetrodotoxin derivatives, the structure of the parent toxin was not self-evident because of its unusual nature. Based upon the added chemical and spectroscopic evidence that tetrodotoxin was a zwitterion and not a lactone (Goto *et al.*, 1965; Woodward, 1964), the configurational formula was deduced as shown in Figure 4.

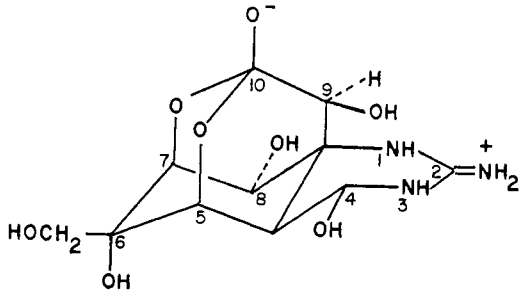

Figure 3. Tetrodotoxin

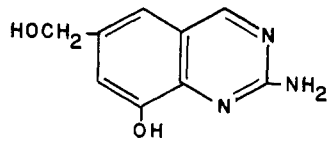

Figure 4. 2-Amino-6-hydroxymethyl-8-hydroxyquinazoline

That the crystalline toxin possessed a dimeric anhydro form with two molecules joined by an ether linkage at C-4 has been disproved, based on the x-ray determined unit cell volume of crystalline tetrodotoxin itself (Woodward and Gougoutas, 1964).

The structure of tetrodotoxin presents several unique features. The cyclic hemilactal (dioxa-adamantane) structure is unknown in any other natural or synthetic product. The zwitterion structure with the guanidinium cation and hemilactal anion is also unique. The low pK_a value for tetrodotoxin was reconciled by the zwitterion structure in which the pK_a value of 8.7 represents the titration of the hemilactal anion moiety and the true guanidine pK_a value is not observed because the molecule decomposes in strong base.

Although the pure crystalline toxin is soluble in dilute acid, it is virtually insoluble in water at pH 7, which is very curious for a molecule bearing so many polar groups. Apparently, this is a property of the crystal structure since the less pure, noncrystalline material is essentially infinitely water soluble.

There is no apparent clue to the biogenetic method of synthesis of tetrodotoxin. The molecule bears no close structural relationship to any other known natural product and there appears to be no readily discernible structural pattern which could be traced to amino acid, carbohydrate, or mevalonic acid precursors. No studies aimed at total synthesis have been reported.

The few chemical modifications of the tetrodotoxin structure, the pharmacology of which has been studied, have been inactive or have shown greatly reduced neurophysiological properties (Deguchi, 1967; Narahashi *et al.*, 1967). Thus, although structure-activity relationships are only speculative, the unique neurotoxic properties of tetrodotoxin probably are associated with

the whole molecule and not with just the guanidine moiety or hemilactal unit (Ranney *et al.*, 1968).

It would be a striking fact if tetrodotoxin occurs only in a single order of fishes and a single family of salamanders. Since many other aquatic animals contain unidentified toxins, the possibility was considered that tetrodotoxin is, in fact, much more widely distributed than the data just presented indicate. Might certain of the unidentified fish toxins be tetrodotoxin? Since tetrodotoxin is found in eggs and ovaries, other poisonous fish eggs were investigated to determine the nature of the toxins they contain.

Gar Roe. Several species of gar are found in the brackish waters of the southeastern United States and the Mississippi basin. In his survey of fishes of the Mississippi, Coker (1930) reports that it is said that the roe is poisonous to man. This appears to be the source of several later statements of a similar kind. While such information may perhaps be rejected as hearsay, very real evidence, in the form of poisoned ichthyologists, is available. Personal communications have been received from three ichthyologists who poisoned themselves and their friends with caviar prepared from gar roe, and one of these even repeated the experience a second time! Netsch and Witt (1962) reported that they fed the roe of the long-nosed gar (*Lepisosteus osseus* Linnaeus) to mice and found that it was indeed toxic.

The frozen roe of a large spotted gar, (*Lepisosteus productus*) taken in Double Bayou, Trinity Bay, Tex., was used for the work reported here. The roe was thawed and homogenized in a Waring blender with an equal volume of water. The resulting mixture was administered by stomach tube to 11 Long-Evans rats weighing 150 to 200 grams. The results (Table III) show that the roe is toxic orally and that the LD_{50} is at least 7 grams per kg. During the period 12 to 48 hours after administration of the roe, the rats became lethargic, had ruffled hair, and, in at least one instance, diarrhea. A portion of the roe was extracted with water at pH 5 and the extract dialyzed. The dialyzates were concentrated by lyophilization and injected intraperitoneally into mice. These extracts were not toxic. This method satisfactorily extracts tetrodotoxin from tissues (Wakley *et al.*, 1966). Thus, the toxin from gar roe is relatively slow-acting and nondialyzable and is clearly different from tetrodotoxin.

Cabezon Roe. Roe from the cabezon or marbled sculpin (*Scorpaenichthys marmoratus* Ayers) was used for most of the experimental work. This fish (Figure 5) is common to most of the Pacific coast of North America. It is a favorite sport fish of skin-divers and is also taken commercially along the coast of California. Although the flesh has an excellent flavor, it is somewhat coarse, and is frequently, but not invariably, light turquoise blue in color and is therefore sometimes rejected by the unsophisticated buyer. Although the flesh is delicious, the roe has been known to be poisonous since 1923—when the ichthyologist Carl Hubbs and his wife became ill after eating roe from a cabezon taken at Point Lobos, Calif. He reported that four hours after eating the roe they awoke in misery with alternating chills and fever, vomiting, and diarrhea. Hubb's experience is apparently the source of statements by Walford (1931) and Schultz (1938) that cabezon roe is poisonous, although it was not until 1951 that Hubbs and Wick (1951) published a note recounting the gastronomic episode together with data showing that ground cabezon roe administered orally produced illness and death in mice and guinea pigs. Pillsbury (1957) observed that on the coast of British Columbia the eggs of the cabezon were avoided by birds, mink, and raccoons. Thus, the eggs of this fish have been observed to be toxic when eaten by man, to produce death when fed to laboratory animals, and to be avoided as food by natural predators.

Figure 5. Cabezon or marbled sculpin: *Scorpaenichthys marmoratus* **(Ayers)**

From Halstead (1967) with permission of the author

MATERIAL AND COLLECTION. On the California coast the cabezon spawns from October to April (O'Connell, 1953). Roe were collected during these months each year since 1964. Fish were obtained from both sport and commercial fisheries from Santa Barbara to Bodega Bay, although most of the experimental work was done with roe from fish caught in Monterey Bay. Roe were frozen as soon as possible after the fish was caught, almost always within 24 hours. In some instances, the eggs were homogenized with an equal volume of water in a Waring blender and then lyophilized. The lyophilized eggs were stored at −20° C. For many experiments, the fresh frozen eggs were homogenized in the Waring blender with twice their volume of 0.5 or 0.9% NaCl, and the homogenate was then centrifuged at 5° C. and about 10,000 G for 10 minutes. The supernatant cloudy fluid is designated the saline extract.

GENERAL EFFECTS OF ADMINISTRATION TO ANIMALS. Administration of homogenized fresh roe, homogenized lyophilized roe, or aqueous or saline extracts of roe to rats or mice, either orally or intraperitoneally, produced a reproducible sequence of signs that, with adequate dosage, resulted in death in 15 to 48 hours (Table IV). Usually the animals showed no evidence of toxicity for at least 12 hours after administration of surely lethal doses. Then they tended to seek corners of the cage, and the fur became ruffled. Subsequently, they became less active, sometimes exhibited diarrhea and nasal dis-

Table III. Toxicity of Roe of the Spotted Gar *Lepisosteus productus* **in Rats**

No. of Animals	Dose (Dry wt.) G./Kg.	Route	Mortality, %	Time to Death, Hr.
9	4	Oral	33	48
2	7	Oral	50	24

Table IV. Toxicity of Aqueous Extracts of Cabezon Roe

Animal	No.	Route	Dose, G. Dry/Kg.	Mortality, %	Time to Death, Hr.
Rat	1	oral	2	0	—
Rat	4	oral	4	75	24
Rat	13	oral	6	77	24-48
Rat	7	oral	8	86	15-18
Rat	2	oral	10	100	15-18
Rat	2	i.p.	4	100	15-18
Mouse	1	oral	4	0	—
Mouse	3	oral	8	66	24
Mouse	5	oral	12	100	18-20
Mouse	1	i.p.	1	100	36
Mouse	5	i.p.	3	80	22-28
Mouse	2	i.p.	4	100	22-24
Mouse	3	i.p.	6	100	20
Mouse	2	i.p.	10	100	20
Mouse	1	i.p.	15	100	18

charge, and eventually became comatose before death. The data in Table IV show a clear trend toward decrease in time between administration of the toxin and death with increase in dose. However, this period could not be reduced to less than about 15 hours even with three to five times the LD_{50}. The slow onset of signs of intoxication was observed with intraperitoneal as well as oral route of administration.

Distribution of the Toxin. Data indicate that the toxin is present in significant quantity only in the ova of the cabezon. In doses of 16 grams per kg., aqueous extracts of liver, testes, and spent ovaries of the cabezon were not toxic when administered intraperitoneally to mice. On a dry weight basis, this dose is at least four times the surely lethal dose of similar extracts of cabezon eggs.

Seasonal and Geographic Differences. All roe tested contained the toxin. Precise comparisons of the amount of toxin in immature and mature roe have not been made, but gross differences in toxicity have not been noted. While most experimental fish have been obtained from Monterey Bay, roe from fish taken off Santa Barbara and near Bodega Bay, roughly 200 miles south and 150 miles north, respectively, of Monterey are also toxic. Further, Hubbs and Wick (1951) found roe from a fish taken off La Jolla (400 miles south of Monterey) to be toxic. Therefore, there is no evidence to suggest that the toxicity of the roe is the result of some local toxic factor in the diet.

Physiological Effects. Since the toxicity data presented were obtained with crude extracts of eggs, it was first necessary to establish that the toxicity was not due to contaminating microorganisms. Saline extracts of cabezon roe were sterilized by passage through a GF Millipore filter. Dry, lyophilized, powdered eggs were sterilized with ethylene oxide, and then dissolved in sterile 0.9% NaCl. Aliquots of these solutions were tested for sterility by inoculation into brain-heart infusion broth. No growth of microorganisms was observed. The sterile solutions produced typical symptoms and death in mice when administered intraperitoneally. Thus it is clear that living microorganisms are not responsible for the toxicity.

Searches for any clearly reproducible acute physiological effects produced by cabezon toxin have been unsuccessful. These negative experiments can be summarized as follows:

Cabezon toxin is not hemolytic on sheep, mouse, or human red blood cells.

The toxin does not itself have proteolytic activity on hemoglobin or on the purified alkaline phosphatase of *Eschericha coli*.

Cabezon toxin injected intradermally in the guinea pig does not increase permeability of the small blood vessels to intravenously injected dye (T-1824).

Cabezon toxin does not affect staining of tissue when present with T-1824 in intestinal loops of the guinea pig.

In three out of five experiments, the toxin increased the permeability of the isolated toad bladder to sucrose when measured with C^{14} labeled sucrose.

Cabezon toxin had no effect on spontaneous contraction of isolated guinea pig ileum or on contractions of the intestine produced by acetylcholine.

The toxin had no effect on smooth muscle of the perfused rabbit ear artery (method of De La Land and Rand, 1965) or on contractions of this muscle produced by stimulation of the peripheral sympathetic nerve.

The toxin produced no acute circulatory effects on heart rate or blood pressure within 8 hours when injected intraperitoneally into rats.

Cabezon toxin in concentrations of 50 mg. of dry eggs per ml. had no effect on axonal conduction in desheathed frog sciatic nerves.

Cabezon toxin did have reproducible, dose-dependent effects on growth of mouse fibroblasts (L-929, Earle, Microbiological Associates) in tissue culture. They were grown in Eagle's minimum essential medium (Joklik Modified, Schwarz BioResearch) containing glutamine and 10% bovine serum, in 4-ounce prescription bottles gassed with 5% CO_2-95% air and incubated at 37° C. Approximately 1.5 to 4 $\times$ 10^5 cells were used per bottle in 15 to 20 ml. of fresh medium. After growth for two to three days (at which time clumps of cells had adhered to the glass), the medium was aseptically removed, and fresh medium with or without cabezon toxin (sterilized by passage through Selas 02 filter) was added. Growth was then followed by measurement of total protein (Oyama and Eagle, 1956), and, in some experiments, by counting the cells, for an additional one to five days. In Figure 6, the results of a typical experiment are plotted as total protein per bottle *vs.* time. Under these conditions, saline extracts of cabezon toxin inhibit growth of mouse fibroblasts in a concentration of 4 mg. dry roe per ml. In other experiments, the inhibitory effect was 10% in three days at a concentration of 0.4 mg. per ml.

Pathology. The acute pathological effects of administration of crude cabezon toxin to mice have been studied in collaboration with Alex M. Saunders of the Department of Pathology, Stanford University, and will be published elsewhere (Saunders and Fuhrman, 1968). In one series of experiments, surely lethal doses (2 grams of dry roe per kg.) were administered intraperitoneally to mice, and after 24 hours the surviving animals (five out of six) were anesthetized with ether and the organs removed and fixed. Grossly, the livers appeared pale and somewhat more friable than normal, and the small intestines were frequently filled with fluid. Micro-

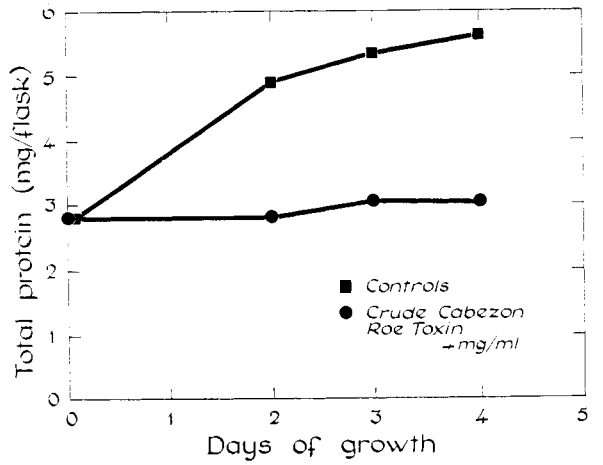

Figure 6. Effect of cabezon toxin on growth of cells in tissue culture

scopically, the livers of all animals showed areas of focal necrosis. In the spleen of all animals that received the toxin, the germinal follicles were farther apart than in controls, and the splenic pulp contained numerous degenerating red blood cells which were undergoing phagocytosis. There was a general decrease in the number of lymphocytes, but megakaryocytes were present in normal numbers.

CHEMICAL PROPERTIES OF THE TOXIN. The toxin from cabezon roe is not dialyzable through Visking tubing and therefore is clearly different from tetrodotoxin. The two toxins are also easily differentiated on the basis of their physiological effects.

The cabezon toxin could not be extracted from dried roe with ether or with chloroform and is therefore not a simple lipid.

The toxicity of saline extracts of cabezon roe was abolished by autoclaving at 120° C. for 10 minutes or by heating at 90° to 95° C. for the same time. Dry preparations of the toxin lost their toxicity in a few weeks at room temperature. The toxin could be readily obtained in solution by extraction of lyophilized or fresh eggs with 0.5 or 0.9% NaCl. It was precipitated from these solutions with acetone, ethanol, or ammonium sulfate, but it could not be separated from proteins of the eggs with these reagents. For example, about 50% of the toxin was precipitated with 10% ethanol (0° C.), but over 40% ethanol was required for complete precipitation, when, of course, the precipitate included most of the solids of the roe extract. When toxic precipitates were obtained with weak ethanol, or by simple dilution of saline extracts with water, these did not readily redissolve in 0.9% sodium chloride. A toxic fibrous material could be precipitated from saline solutions of the crude toxin by simple 10-fold dilution with distilled water. However, this represented only a part of the total toxicity of the original solution. The toxicity is not destroyed by incubation with ribonuclease A (Worthington Biochemical Corp.) or bovine pancreatic ribonuclease (Armour Laboratories) and does not therefore appear to be a ribonucleic acid.

Two methods have yielded the most toxic fractions:

Precipitation. Frozen cabezon eggs were mixed with two volumes of cold 0.14*M* NaCl, homogenized in a Waring blender, and left overnight at 4° C. The suspension was then centrifuged, the precipitate discarded, and the supernatant brought to pH 4.25 with 1*M* acetic acid. This was left overnight at 4° C., centrifuged, and the supernatant discarded. The precipitate was dissolved in 0.14*M* NaCl, brought to pH 6.9 with 1*M* NaOH, and dry NaCl was added to a final concentration of 180 grams per liter. After standing 48 hours at 4° C., the precipitate was collected by filtration and dialyzed against distilled water. The yield from 1500 grams of fresh eggs was 10.5 grams of light tan fluffy powder that gave on analysis C = 57.57%, N = 6.70%, H = 8.27%, and P = 0.85%. The LD_{50} by intraperitoneal injection into mice was about 150 mg. per kg., and the total amount of toxin recovered in the precipitate was 15% of that present in the original mass of eggs.

Column Chromatography. Frozen cabezon eggs were mixed with two volumes of 0.5% NaCl and homogenized in a Waring blender. The mixture was centrifuged at 5° C. and the supernatant liquid decanted. A volume of 6 to 7 ml. of this was applied to a 2.1 × 45 cm. polyacrylamide column (Biogel P-2, BioRad Laboratories) that had been hydrated with 0.5% NaCl. Elution was carried out with 0.5% NaCl at 4° C. at a rate of 1 ml. per minute. The first void volume (65 ml.) was discarded. Successive fractions of approximately 10 ml. (B, C, D, etc.) were collected and assayed for toxicity. Maximum toxicity was always associated with the third fraction after the void volume (Fraction D), although C and E were also found to be toxic. In a typical experiment, the original supernatant (equivalent to 2.7 grams of fresh roe) containing 965 mg. of dry material, (exclusive of the added salt) was placed on a column. The dry weight of Fraction D was 268 mg. This gave an ultraviolet spectrum with a shoulder at 270 to 280 mμ. (not very different from the starting material), and assayed C = 49.3%, H = 7.4%, N = 11.6%, and P = 0.61%. The LD_{50} determined by intraperitoneal injection into mice was about 200 mg. per kg. The dry material of Fraction D contained 1.4% fat extractable with cold chloroform. This fat was not toxic when mixed with almond oil and injected intraperitoneally into a mouse.

In other experiments, many other methods for extraction of the toxin were tried with less success than those just described. About 10% of the toxin could be extracted from dry lyophilized roe with isopropanol. Other solvents were less effective. Chromatography on columns of other materials (Biogels P-6, P-10, A-0.5, and A-1.5) was tried without success.

The chemical properties of the toxin from cabezon roe indicate that it is a protein or that it is a compound bound to a protein in the native state.

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