Tarichatoxin—Tetrodotoxin: A Potent Neurotoxin

A nonprotein substance isolated from the California newt is the same as the toxin from the puffer fish.

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In literature and alchemy the salamander is a fabled creature. Salamanders have long been known to be toxic animals, the poisons they produce usually being associated with secretions of specialized glands or of the skin itself. An extraordinarily powerful neurotoxin, called tarichatoxin, has recently been isolated in crystalline form from the eggs of various species of western American newts of the genus *Taricha*. This toxin, present in adult newts as well as in newt eggs and embryos, is very different chemically and pharmacologically from other known salamander toxins. This makes all the more remarkable the finding, in recent work, that tarichatoxin is identical to a toxin known as tetrodotoxin which occurs in the Japanese Fugu or puffer fish. The substance appears to occur only in one family of Amphibia (the Salamandridae) and one suborder of fishes (the Tetraodontoidae). This extremely limited distribution is a remarkable biogenetic finding.

Here we discuss the course of the investigations which led to the isolation of the toxin from the California newt, the history of tetrodotoxin, the evidence, both physical and physiological, which points to the conclusion that tarichatoxin and tetrodotoxin are one and the same substance, and the deductions which can be made concerning the chemical structure of the toxin.

Tarichatoxin

Discovery and history. In the early 1930's Victor C. Twitty, an experimental embryologist, came to Stanford University from Yale. At New Haven he had worked with the eastern salamander, *Ambystoma punctatum*, and at Stanford he began looking for a suitable, locally abundant, substitute. He soon found that the California newt, *Taricha torosa* (formerly *Triturus torosus*) (Fig. 1), was especially abundant near the Stanford campus in the early spring, when it came down from the hills to spawn in nearby ponds and streams. This species met his needs admirably.

In the course of his studies Twitty made the surprising incidental observation that when the eye vesicles of *Taricha torosa* were transplanted into an embryo of *Ambystoma tigrinum*, the tiger salamander, the host became paralyzed and remained so for days (*2*). The paralysis was temporary, the host and graft were otherwise normal, and no signs of developmental retardation or injury were observed. Subsequently, embryos of the two species were joined as parabiotic twins (*3*); on stimulation, the *A. tigrinum* member of the pair was found to be paralyzed, but the *T. torosa* member was normal. In detailed experiments Twitty (*4*) showed that the toxin was present in all tissues of *T. torosa* embryos and in the blood of some adult females. He concluded that the embryos of *Taricha* must contain a toxic substance capable of paralyzing *Ambystoma*.

The concentration of the toxin in extracts of eggs and embryos could be estimated by injecting the toxin into mice and observing the survival time (*5*). One "mouse unit" was defined as the amount of toxin, contained in a 0.2-milliliter volume, which would kill a 20-gram mouse in 10 minutes when injected subcutaneously. On this basis a reliable bioassay for the toxin was developed (*5*). We used this method of bioassay routinely during isolation of the toxin. Whereas the crude dried residue from the expressed juice of *Taricha torosa* embryos contained about 3 mouse units of the toxin per milligram, the best fractions obtained in the early work in 1940–1942 contained about 75 mouse units per milligram (*5, 6*). When the crude toxin was injected into mammals, even in exceedingly small amounts, it produced paralysis of skeletal muscle, marked fall in blood pressure, respiratory arrest, and death. We now know that the best preparation obtained in 1940–1942 contained about 1 percent of crystalline toxin, which has a toxic activity equivalent to about 7000 mouse units per milligram—that is, 1 gram of the toxin constitutes approximately 7 million lethal doses (lethal for 20-gram mice within 10 minutes after subcutaneous injection). If the same toxicity for man is assumed, the lethal dose of toxin (injected subcutaneously) for a 70-kilogram man would be about 0.5 milligram. The toxicity in relation to that of other well-known poisons is given in Table 1.

Isolation and purification. Since tarichatoxin is present in the fresh egg clusters of *Taricha torosa* to the extent of only about 3 to 4 parts per million, a considerable supply of eggs was required in these studies. *Taricha torosa* lays its egg clusters (Fig. 1 and cover) in the early spring, the exact time being greatly dependent upon the winter rains. The egg clusters are laid under the water, either attached to branches, twigs, or reeds along the banks or free along the bottom of the ponds, in depths ranging from a few centimeters to 1½ meters and possibly more. Collecting the clusters from the bottom of a pond with mud banks is very difficult, especially when the rains are heavy and the water is deep and roily. Fortunately one collection site, on the Moreshead estate, about 8 kilometers from the Stanford campus, is a reservoir with a cement bottom. The rains were very light during the early winter of 1960–61, and from 8 to 25 February, 1961, about 100 kilograms of eggs were collected by hand, with a kitchen strainer, from the very shallow waters of this pond (*7*). Since each egg cluster weighs, on the average, about 7.5 grams and contains about 20 embryos, the 100 kilograms of eggs corresponds to approximately 13,000 egg clusters or about ¼ million individual embryos.

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Bioassay shows that this quantity of eggs contains about 330 milligrams of pure toxin.

In *Taricha torosa* the interval between the laying of the egg clusters and the time when the capsule that surrounds the embryos disintegrates and the larvae swim free is 2 to 3 weeks; thus, the period during which the eggs can be successfully collected in any quantity is limited. The effect upon the newt population of egg collection on such a large scale is not known, but one pond on the Moreshead estate has yielded approximately 100 kilograms of eggs annually for three successive years. As the young female probably does not return to spawn until the third year or later (*1*), no effect of these large collections would be noticeable yet. *Taricha torosa sierrae*, probably identical to *T. torosa* in all respects except range, lays its eggs in streams and ponds of the California Sierra Nevada foothills later in the spring, and about 40 kilograms of these eggs were collected in April of 1962.

Collecting a quantity of eggs of the western red-bellied newt, *Taricha rivularis*, was much more difficult. In this species egg clusters (Fig. 2) are laid where they become attached to the undersides of rather large stones in rapidly flowing currents of streams in the coastal mountains north of San Francisco Bay (*1*). Collecting the clusters requires much physical labor, since the stones must be lifted and turned. In late March of 1962 about 16 kilograms of the clusters were collected.

The rough-skinned newt, *Taricha granulosa*, lays its eggs singly and over a longer period (from May through August) in Northern California, Western Oregon, and Washington. No attempt was made to collect enough material for isolation of the toxin from this source, but it was determined that the eggs are toxic.

Many methods for isolating the toxin have been explored; the most successful was that developed by M. S. Brown on the batch of *Taricha torosa* eggs collected in 1961 (*8*). At every step of the separation process there was subcutaneous injection of the various fractions into mice. Without this reliable assay it would have been quite impossible to have isolated this trace constituent from the egg clusters. The procedure is shown schematically in Fig. 3. The first few steps, through dialysis, are carried out according to a technique developed by Horsburgh, Tatum, and Hall (*5*) in 1940 and by van Wagtendonk, Fuhrman, Tatum, and Field (*6*) in 1942. The fact that the toxic principle was dialyzable showed that it is nonprotein and thus chemically distinct from many known animal venoms. Purification beyond the stage of 10 to 15 mouse units per milligram proceeded very slowly, and the process required, in all, more than 2 years.

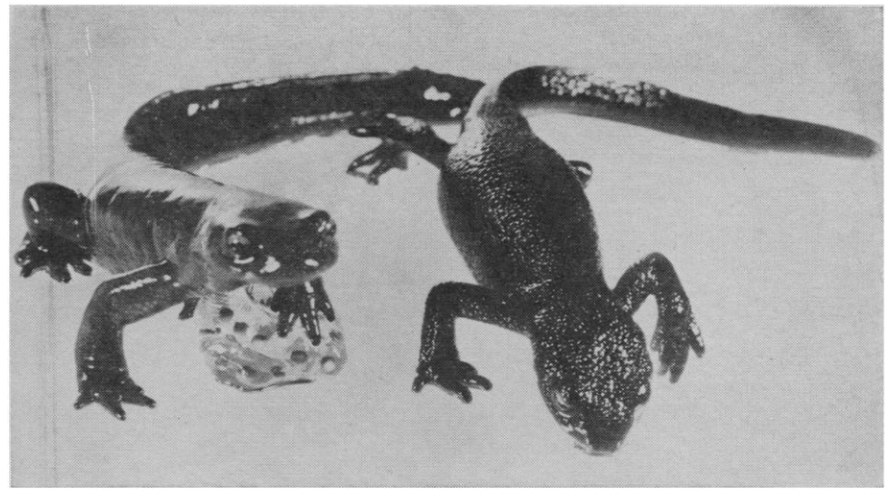


Fig. 1. *Taricha torosa*, male, and female with single egg clusters.

For many months we were unable to achieve a degree of purity better than about 3000 mouse units of toxin per milligram (that is, purity of about 40 percent). By that time an assay technique of thin-layer plate chromatography had been devised which involved development on Merck silica gel G with acetic acid (4 percent) in absolute ethanol. Upon spraying the plate with base, the toxic material was found to correspond to a location that showed as a yellow fluorescent spot (R_F, 0.39) (see Fig. 4). It was not successfully separated by column chromatography or electrophoresis. Eventually we achieved some further purification by dissolving the partially purified toxin (activity of 3000 to 5000 mouse units per milligram) in dilute acetic acid and then neutralizing with pyridine. We were at last rewarded with microcrystals when ether was added to a solution of the impure toxin in 5-percent ethanolic acetic acid. These

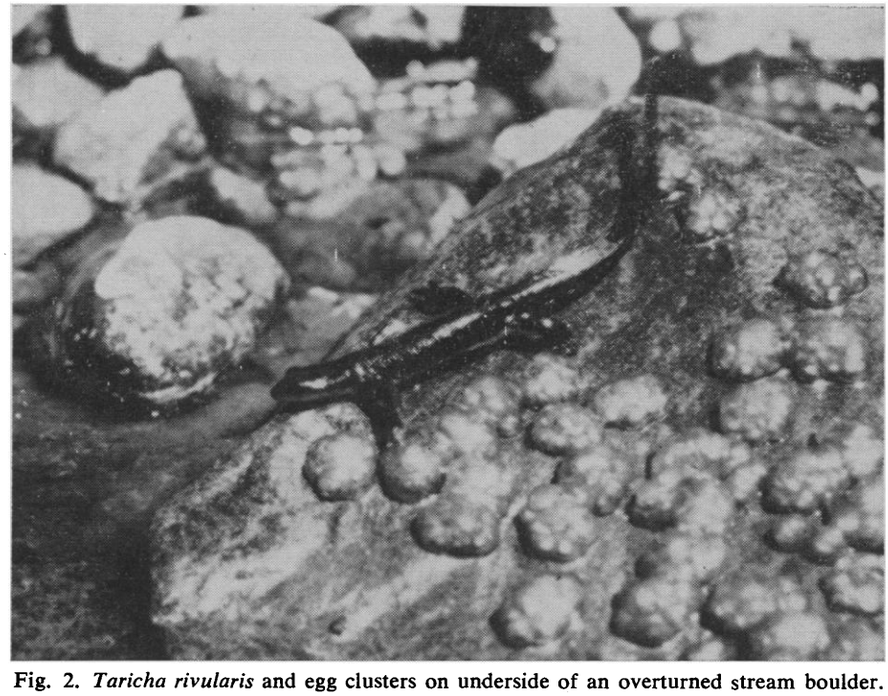


Fig. 2. *Taricha rivularis* and egg clusters on underside of an overturned stream boulder.

crystals had a toxic activity of about 7000 mouse units per milligram. From the first 100 kilograms of eggs we obtained about 200 milligrams of crude material with an activity of about 3000 mouse units per milligram. The major portion of this material was converted into a series of acetate derivatives (*8*); 12 milligrams of crystalline toxin were ultimately obtained from the remainder.

We have now found that the most satisfactory method of final purification is to pass carbon dioxide through an aqueous suspension of the toxin until it dissolves (a 0.1-percent solution of the toxin can be obtained), remove any insoluble material by centrifugation, and allow the saturated carbon dioxide solution to stand in a closed container over a 0.01-percent solution of ammonium hydroxide. A microcrystalline product slowly forms near the surface of the solution.

Unknown variables and the instability of the toxin have rendered the reproducibility of this whole procedure uncertain, especially at the stage of silicic acid chromatography. It is remarkable that in the stages through chromatography the toxin is always found in the alcohol-soluble and water-soluble fractions, yet that, after electrophoresis and evaporation, the toxin is almost completely water- and alcohol-*insoluble*. In fact, the toxin in a relatively pure form dissolves only in acidic solutions. If the acid solution is too strong, decomposition is rapid and toxic activity is lost. Even though the toxin appears to be insoluble in basic solution, it is rapidly destroyed in such solutions. The purified toxin shows no solubility in dimethyl formamide, acetonitrile, or dimethylsulfoxide or in the usual organic solvents.

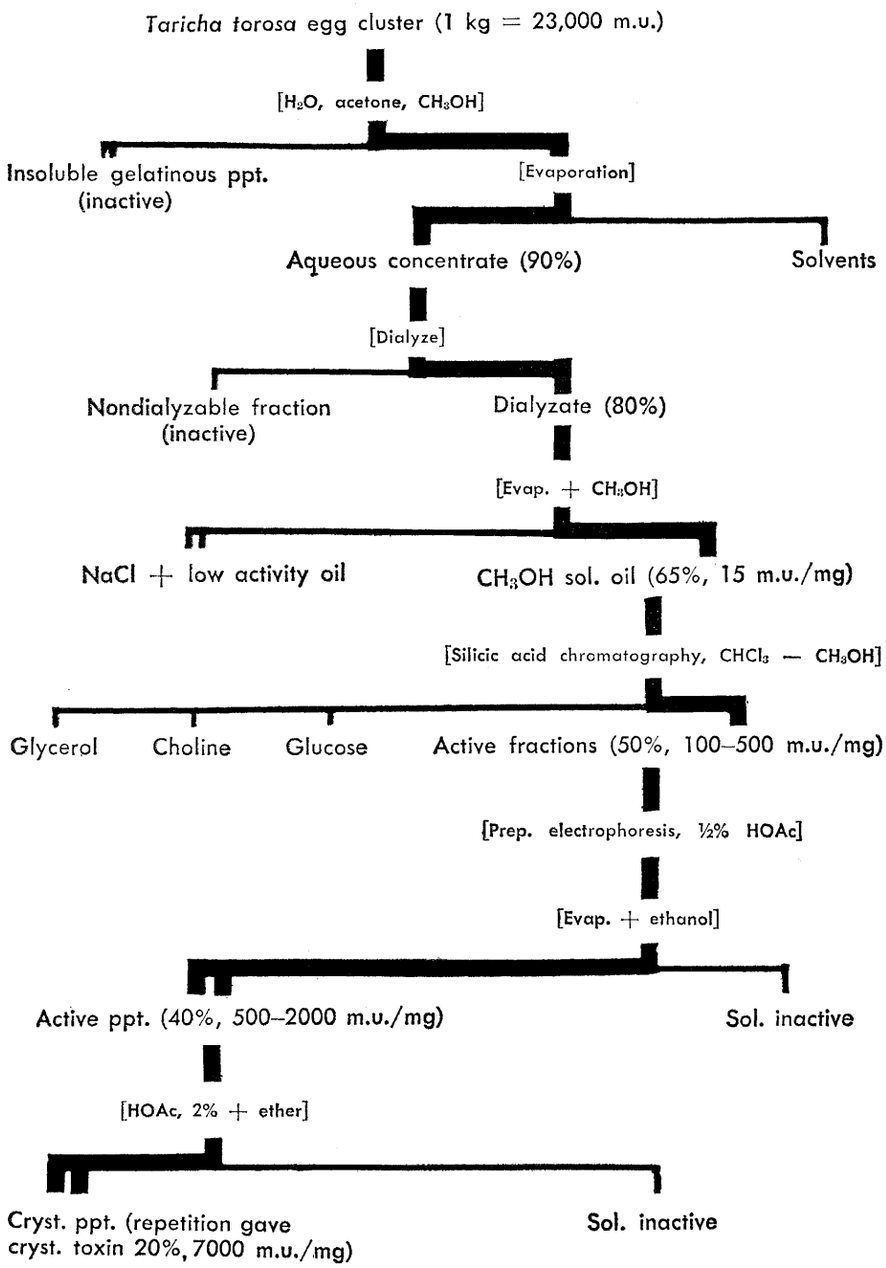


Fig. 3. Schematic diagram of the isolation procedure. The mouse units per milligram (m.u./mg) are average values based on the composite of several isolations. The approximate cumulative efficiency of recovery is indicated by a percentage and is based on the average of several of the more favorable separations.

With this isolation procedure enough toxin was obtained from the eggs of *Taricha rivularis* and *T. torosa sierrae* to show, on chemical as well as pharmacological grounds, that the toxins of these two species are identical with that of *T. torosa*.

As the toxicity of the preparations of tarichatoxin increased during purification it became apparent that we were dealing with a substance that was among the most potent nonprotein animal or plant toxins known (Table 1). Comparisons were therefore made with other highly toxic substances. Saxitoxin, the shellfish poison (*9*), has the same order of toxicity as tarichatoxin but clearly differs from it in both chemical and pharmacological properties. Tetrodotoxin, isolated from the ovaries and liver of various tetraodontoid fishes known as Fugu or puffers (*9*, *10*) (Fig. (5), was found to have about the same toxicity as the best preparations of tarichatoxin. [Kokoi venom (*11*), slightly more toxic than tarichatoxin, had not been described at this time.] As the isolation of tarichatoxin progressed, the chemical and pharmacological findings began to force us toward what would initially have seemed a very unlikely conclusion—that tarichatoxin from the California newt is the same substance as tetrodotoxin from puffer fish. This conclusion was reinforced by the finding that tarichatoxin (*12*), like tetrodotoxin (*13*), is about 40 to 50 times as toxic when administered parenterally as when ingested orally.

In view of this apparent identity, let us turn back in this account to consider the toxin known as tetrodotoxin and the puffer and other fish of the suborder Tetraodontoidae that are its source. We begin with the historical background.

Tetrodotoxin:

Its History and Importance

Like tales of the salamander, pictures and stories of the puffer fish go back to antiquity. The puffer *Tetraodon lineatus* has been identified in figures on the tomb of the Egyptian pharaoh Ti (*14*) of the Vth Dynasty (2500 B.C.), and it is believed that the early Egyptians knew the puffer to be poisonous (*15*).

Iwao Tani (*16*), in his definitive monograph on the occurrence of toxicity among the tetraodontoid fishes, summarized some of the ancient writings on Fugu poison. His earliest reference is a writing from the Han dynasty (202 B.C.–A.D. 220) which may be translated "Salmon liver kills a man." It is presumed that the so-called "salmon" in ancient Chinese writings is the Fugu. In a Chinese treatise by Chaun Yanfang, "Studies on the Origins of Diseases," written during the Sui Dynasty (A.D. 581–617), an accurate account is given of the toxicity of the liver, eggs, and ovaries of a fish which seems, from its description, to have been the Fugu.

Kaempfer, in his well-known *History of Japan* (*17*) based on his experiences while Physician to the Dutch embassy to the Emperor's Court in Japan in 1690–92, relates in some detail the toxic properties of three varieties of fish called "Furube" by the Japanese. "The Dutch call him Blazer which signifies Blower, because he can blow or swell himself up in the form of a round ball. He is ranked among the poisonous Fish, and if eat whole is said unavoidably to occasion death. This [Mabuku] the Japanese reckon a very delicate Fish and they are very fond of it. But the head, guts, bones and all the garbage must be thrown away and the flesh carefully washed and cleaned before it is fit to eat. And yet many people die of it, for want, as they say, of thoroughly washing and cleaning it. People that by some long and tedious sickness are grown weary of their lives, or are otherwise under miserable circumstances, frequently choose this poisonous fish, instead of knife or halter, to make away with themselves."

Puffer-fish poisoning is not limited to Japan and the Middle East; the early Spanish padres in Mexico also encountered it.

In an account of the search for a new mission site in Baja California, the Mexican historian Francisco Javier Clavijero records (*18*) that four soldiers found a bonfire in which native fishermen had roasted and eaten some *"botete"* (*Sphoeroides lobatus*) but had left the liver on some shells. In spite of urgent warning by one of the natives, one of the soldiers began to eat it and to share it with the other three. One of them ate a little; another only chewed it without swallowing it; the third only touched it. The first died in half an hour, the second shortly afterward, the third remained unconcious until the following day.

Almost two centuries after Clavijero, John Steinbeck and E. F. Rickets visited Baja California and described their voyage in *Sea of Cortez* (*19*). They offered to buy a *botete* from a boy in La Paz, but the boy refused, "saying that a man had commissioned him to get this fish and he was to receive ten centavos for it because the man wanted to poison a cat."

There have been recent incidents of poisoning by puffers (*Sphoeroides testudineus*) in Florida (*20*), and toxic puffers have been described in the coastal waters of the United States as far north as New England (*21*). However, it is chiefly in Japan that puffer-fish poisoning is a major public health problem. Recent data collected by the Japanese Ministry of Welfare (*22*) show that in the period 1956–58, 715 individuals were poisoned from eating puffer fish; of these, 420, or 59 percent, died. Regulations now require the licensing of cooks who prepare puffer fish for eating, in certain prefectures in Japan, and forbid the sale of viscera in others (*22*). However, control is apparently not simple, since puffers are a favorite food and the viscera are said to give a desirable flavor (*23*).

Understandably, much of the work on tetrodotoxin has been done by Japanese scientists, and it was to one of them that we turned for material for our studies.

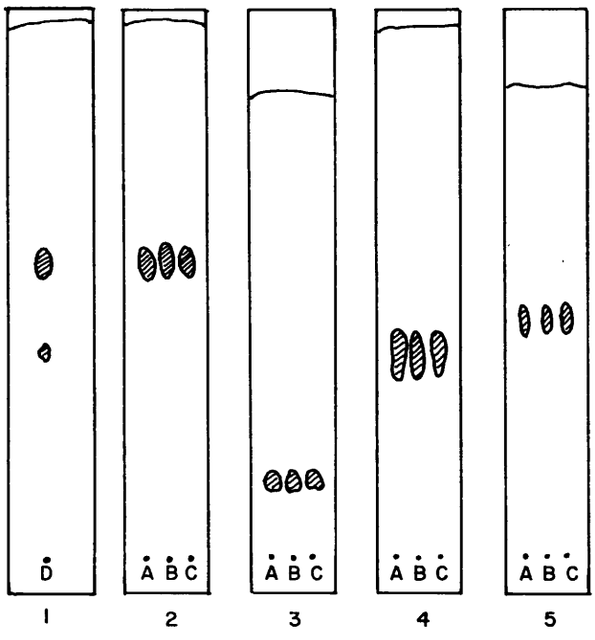
Identity of the Toxins

As soon as we had obtained crystalline tarichatoxin we entered into correspondence with K. Tsuda of the Institute of Applied Microbiology of

Table 1. Relative toxicities of a selected group of toxic substances.

Toxin	Minimum lethal dose (μg/kg)*	Source	Form and/or structure	Molecular weight	Reference
		Protein			
Botulinus toxin A	0.00003	Bacterium: *Clostridium botulinum*	Crystalline, A	900,000	(*61*)
Tetanus toxin	.0001	Bacterium: *Clostridium tetani*	Crystalline	100,000	(*61*)
Ricin	.02	Plant: castor bean, *Ricinus communis*			(*62*)
Diphtheria toxin	.3	Bacterium: *Corynebacterium diphtheriae*		72,000	
Cobra neurotoxin	.3	Snake: *Naja naja*			(*62*)
Crotalus toxin	.2	Snake: rattlesnake, *Crotalus atrox*			(*62*)
		Nonprotein			
Kokoi venom	2.7†	Frog: *Phyllobates bicolor*		~400	(*11*)
Tarichatoxin	8	Newt: *Taricha torosa*	$(C_{11}H_{17}N_3O_8)$	319	(12)
Tetrodotoxin	8–20	Fish: *Sphoeroides rubripes*	$(C_{11}H_{17}N_3O_8)$	319	(*9, 10*)
Saxitoxin	9	Shellfish. Produced by dinoflagellate *Gonyaulax catenella* ingested by shellfish	$(C_{10}H_{17}N_7O_4 \cdot 2HCl)$	372	(*9*)
Bufotoxin	390	Toad: *Bufo vulgaris*	Vulgarobufotoxin $(C_{40}H_{60}N_4O_{10})$	757	(*62*)
Curare	500	Plant: *Chondodendron tomentosum*	*d*-Tubocurarine $(C_{38}H_{44}N_2O_6Cl_2)$	696	(*62*)
Strychnine	500	Plant: *Strychnos nux-vomica*	$(C_{21}H_{22}N_2O_2)$	334	(*62*)
Muscarin	1,100	Mushroom: *Amanita muscaria*	$(C_9H_{20}O_2NCl)$	210	
Samandarin	1,500	Salamander: *Salamandra maculosa*	$(C_{19}H_{23}O_2N)$	397	(*33*)
Diisopropyl-fluorophosphate	3,000		Synthetic nerve gas $[(C_3H_7)_2PO_3F]$	184	
Sodium cyanide	10,000		Synthetic NaCN	49	(*62*)

* Minimum lethal dose refers to mouse except in the case of ricin, where it refers to guinea pig (see *61*), and of bufotoxin and muscarin, where it refers to cat. In cat, administration was intravenous; in all other cases it was intraperitoneal. Since the survival times are not always specified and the experiments are not direct comparisons, these values are of necessity approximate and indicative only of relative toxicity by the indicated route of administration. † LD_{50} in mouse, administered intravenously.



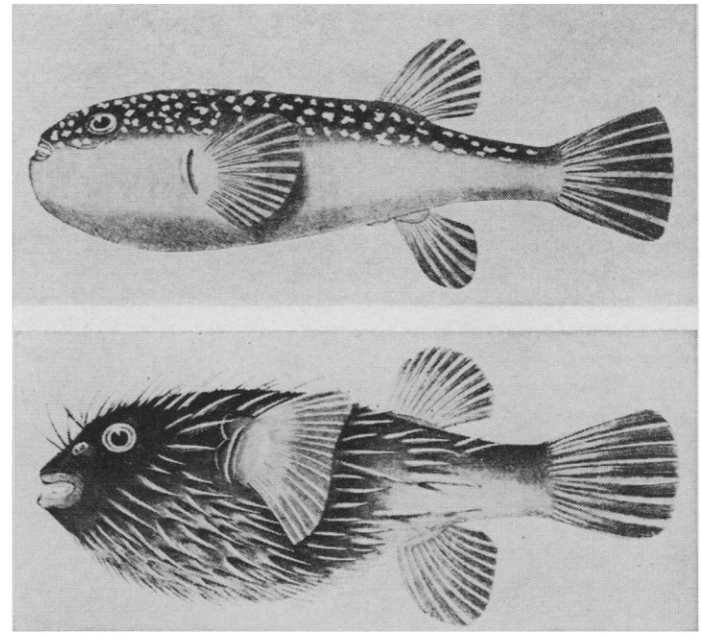


Fig. 4 (left). Thin-layer chromatography (silica gel G, Merck) of tarichatoxin and tetrodotoxin. (Plate 1) The two spots given by a somewhat impure sample. The faster-moving spot is the active toxin. All plates were developed by spraying with alcoholic potassium hydroxide and heating at 130°C for 10 minutes; they were viewed under ultraviolet light. (Plates 2–5) Comparisons of tetrodotoxin A, tarichatoxin-tetrodotoxin mixture B, and tarichatoxin C. Plates 1 and 2 were developed with a mixture of ethanol and acetic acid (96:4); plate 3 was developed with *n*-butanol, acetic acid, and water (50:3:10); plate 4 was developed with collidine and water (50:20); and plate 5 was developed with phenol, ammonia, and water (13:1.8:22). Fig. 5. Pufferfish: (top right) *Tetradon honckenyi* (Bloch) and (bottom right) *Diodon hystrix* (Linnaeus).

the University of Tokyo, who generously supplied us with samples of tetrodotoxin for direct comparison. It was immediately apparent that the two toxins were the same or, at most, differed only in some small detail. The unequivocal proof of identity was rendered especially difficult by the insolubility of the toxin in any but acid solvents, and the fact that it was not indefinitely stable in these, even when very dilute acetic acid was used. In addition, the toxin decomposed without melting. The derivatives that we obtained—namely, acetates—appeared, from spectroscopic evidence, to be structurally different from the toxin itself. These acetate derivatives had no demonstrable toxicity, and proof of the structural identity of such acetate derivatives of the two toxins could not be taken as conclusively demonstrating complete structural identity of the parent toxins.

By comparing the physical properties of the heptaacetate (*24*) derivatives from the two toxins—the melting points, mass spectra, proton nuclear-magnetic-resonance spectra (Figs. 6 and 7), infrared spectra (Fig. 8), and optical rotations—it was established that the derivatives from tarichatoxin and tetrodotoxin were in fact indisinguishable (*25*). Furthermore, the data on a common pentaacetate derivative (*24*) as well as a diacetate derivative (*25*) (the latter prepared according to a method described to us by K. Tsuda) were also identical.

The nonvolatility of the toxins themselves made it impossible to make a meaningful direct comparison of the mass spectra of the two. Furthermore, the nuclear-magnetic-resonance spectra (*25*), although they were identical, were of such a nature that a small difference—for instance, in the stereochemistry of one hydroxyl group—very probably would not be detected. Tetrodotoxin shows no ultraviolet maximum above 220 mμ. We found this to be true also for tarichatoxin, and upon further study we found that both toxins showed a maximum, at *p*H 7 in water, at 197 mμ. The first infrared spectra in potassium bromide windows were not identical, but this proved to be a matter of purity, and ultimately the spectra of the toxins from the two sources were shown to be superposable (*25*). The rich and detailed nature of these spectra make it very unlikely that the two toxins differ even in some small stereochemical detail.

The toxins from the two sources also showed the same chromatographic behavior on direct comparison by thin-layer chromatography, with four different solvent systems (Fig. 4).

The conclusion that the two toxins are identical, arrived at on the basis of the physical evidence cited, is dramatically reinforced by physiological evidence. Both tarichatoxin and tetrodotoxin have an LD_{50}, on intraperitoneal injection into mice, of about 8 to 10 μg/kg (*10, 12*). A dose of about 15 μg of either toxin per kilogram is sufficient to kill mice, rats, frogs, goldfish, and tiger salamanders (*Amblystoma tigrinum*) within a few minutes. However, when doses as high as 1000 μg of either tarichatoxin or tetrodotoxin per kilogram were injected into *Taricha* the animals were neither killed nor paralyzed and appeared completely normal.

Tarichatoxin and tetrodotoxin both block the action potential of desheathed frog nerves in a few minutes when the toxins are applied in concentrations of 1 to 10 μg/liter, but each of the toxins produced only partial block of desheathed sciatic nerves from *Taricha granulosa* when these nerves were treated with a concentration of 30,000 μg/liter for 20 to 30 minutes (*12, 25*). This ability of *Taricha* to tolerate a concentration of toxin which is many times the lethal concentration for other amphibia, mammals, and fish is a remarkable phenomenon which must be studied further.

Occurrence and Distribution

Among the fishes, only members of the suborder Tetraodontoidae have been shown to contain tetrodotoxin. Most toxic species belong to the families Tetraodontidae and Lagocephalidae. These fishes are known as puffers, globefish, swellfish, porcupine fish, and *maki-maki*. Tani (*16*) lists 15 species which were found to be toxic, most of them of the genus *Sphoeroides*. In the central Pacific most poisonous puffers belong to the genus *Arothron*. The highest concentration of toxin is found in the ovaries, and the amount found varies with the season of the year. It is greatest just before spawning in the spring. Lesser concentrations of toxin are found in the liver, intestines, and skin. In a few species the muscle is also toxic.

Among the Amphibia tarichatoxin is found only in the family Salamandridae. Twitty (*4*) showed by transplantation experiments that the toxin was present in several newts other than *Taricha torosa*. We have isolated the crystalline toxin from egg clusters of *T. torosa*, *T. torosa sierrae*, and *T. rivularis* and have found it to be present in eggs from *T. granulosa*. We have sought the toxin in other newts, using a simple chemical separation. Whole animals were decapitated and homogenized in a Waring Blendor. The homogenate was acidified to *p*H 5 and dialyzed twice, at 3°C, against distilled water. The combined dialyzates were concentrated by freeze-drying and injected into mice. The presence of tarichatoxin was indicated by the characteristic effect of the extracts—weakness of the hind limbs, followed by convulsions and death. By this method we have confirmed the results of Twitty's (*4*) transplantation experiments, which showed that toxin was present, in low concentrations, in *Diemictylus viridescens* (formerly *Triturus viridescens*), the red-spotted newt from the eastern United States, and in *Triturus pyrroghaster* from Japan. In addition, toxic extracts were obtained from *Cynops ensicauda* (formerly *Triturus ensicauda*) collected in the Ryukyu Islands (*26*) and from *Triturus marmoratus* collected in Italy. We have not obtained toxic extracts from any other amphibia. We have examined specimens from six families of the order Caudata (Urodela) and have found that the toxic activity of extracts prepared as described earlier is confined to a single family, the Salamandridae. Adults of the following amphibia failed to yield toxic extracts: mud puppy (*Necturus maculatus*), Congo eel (*Amphiuma*), siren (*Siren lacertina*), and lungless salamanders (*Ensatina eschscholtzi; Batrachoseps attenuatus; Aneides lugubris*).

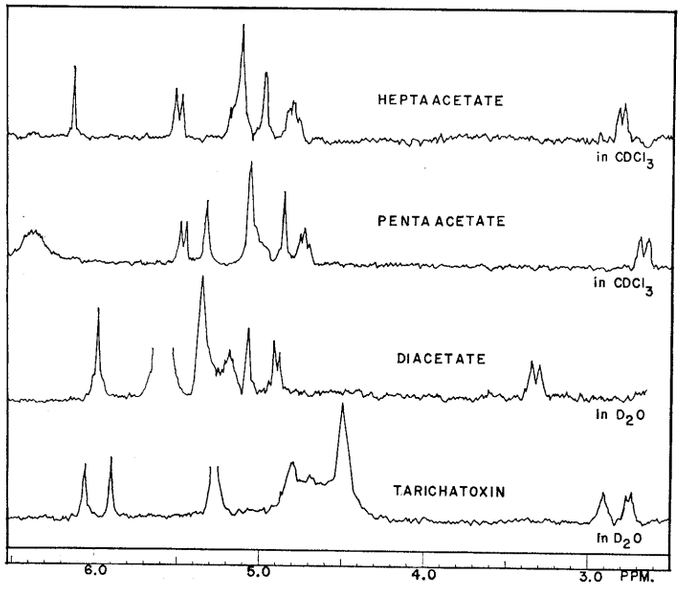
The occurrence of tarichatoxin only in closely related newts now classified in three different genera may be of taxonomic importance and suggests that their classification might well be reconsidered. It is of interest that all these newts were once classified as belonging to the genus *Triturus*.

We once considered the possibility that tarichatoxin is produced by a symbiote rather than by the newt, in the way that saxitoxin is produced not by the mussel or clam but by a dinoflagellate (*9*). This possibility now seems extremely remote, since all specimens of *Taricha* are invariably toxic. This is true even of specimens of *T. torosa* collected at Camp Pendleton, San Diego County, California, in the extreme southern part of their range (*27*).

The distribution of tarichatoxin in the tissues of adult salamanders is now under study. Our results clearly demonstrate the presence of tarichatoxin in adult newts, a result at variance with the results of Twitty's transplantation experiments (*4*).

One can only speculate concerning the function of this toxin in the newt and fish. The hypothesis that it is of survival value, from an evolutionary standpoint, does not seem unreasonable. For instance, catfish had been planted in one of the ponds where the eggs of *Taricha torosa* were collected, and during the spawning season numerous dead catfish were observed floating in this pond.

A more direct indication was the observation by Charles Shaw, of the San Diego Zoological Gardens, that eastern snakes, when offered a *T.*



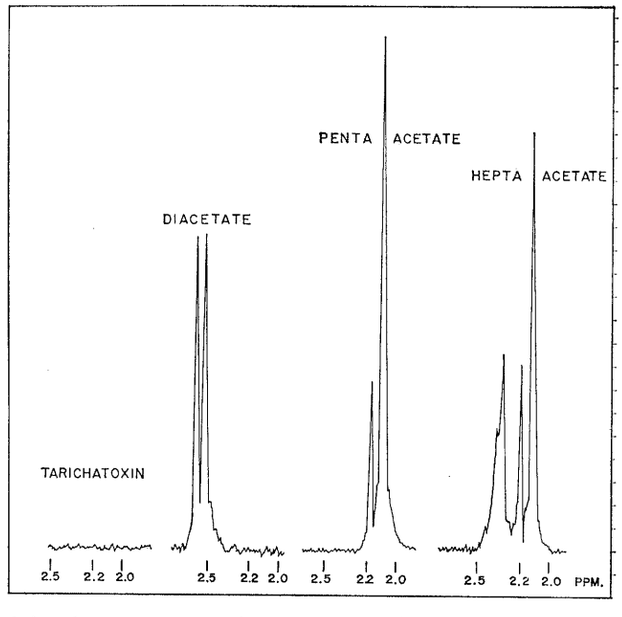


Fig. 6 (left). Nuclear-magnetic-resonance spectra of tarichatoxin and its three acetates. The spectra of the hepta- and penta-acetates were taken in deuteriochloroform, those of the toxin and diacetate, in 10 percent perdeuterioacetic acid in deuterium oxide. The corresponding spectra of tetrodotoxin and its acetate derivatives were identical to the spectra shown. Fig. 7 (right). Nuclear-magnetic-resonance spectra of tarichatoxin and its three acetates in the C-methyl region.

torosa, ate it and died but that western snakes would not accept this newt as food. John Steinbeck's speculations (*19*) about the function of tetrodotoxin in puffers might apply equally well to newts: "*Botete* is sluggish, fairly slow, unarmored, and not very clever at either concealment, escape, or attack. It is amusing but valueless to speculate anthropomorphically in the chicken-egg manner regarding the relationship between his habits and his poison. Did he develop poison in his flesh as a protection in lieu of speed and cleverness, or being poisonous and quite unattractive, was he able to 'let himself go', to abandon speed and cleverness? The protected human soon loses his power of defense and attack. Perhaps *botete*, needing neither brains nor tricks nor technique to protect himself except from a man who wants to poison a cat, has become a frump."

Relationship to Other Toxins

The finding of an identical toxin in tetraodontoid fishes and in newts suggests that this toxin may also occur in other animals, especially in other fish and amphibia. However, this does not appear probable on the basis of our present knowledge. Tetrodotoxin differs from most other fish toxins in that concentrations are high in the ovaries, liver, and skin, rather than in muscle, but this does not eliminate the possibility that location of the toxin within the body varies with species. There are enough differences in the nature of the symptoms, or in their time of onset, in poisoning by puffers and in poisoning by lampreys, hagfishes, elasmobranchs (sharks, rays), scombroid fishes (tuna, skipjack), gempylids, and cabezone to make it highly improbable that the toxin in the latter fishes is tetrodotoxin (*15, 28, 29*). The fact that fishes that cause ciguatera poisoning are toxic in one locality and not in another suggests that the toxin originates in a food chain. The symptoms of poisoning by moray eels (*28*) and ratfish (*15*) and the fact that in the ratfish the toxin is localized in the ovaries suggest that these toxins may be related to tetrodotoxin, but unfortunately nothing is known about the chemical properties of the causative agents in these species.

Many toxins have been reported in various salamanders (*30*). A few are particularly pertinent. Matsusake and Kabeda (*31*) reported a neurotoxin, with some resemblance to tarichatoxin, in the skin of *Triturus* (*Triton*) *pyrroghaster*. Stuhr (*32*) showed that secretions of the skin of *Taricha torosa* were toxic to mammals and produced profound respiratory depression. It is quite clear that tarichatoxin is different chemically and pharmacologically from the well-characterized toxins such as samandarin, samandarone, and samandaridine that have been isolated from specialized glands of *Salamandra maculosa* (*33*), and different from less-well-characterized toxins from the skin of *Triturus taeniatas* and *T. cristatus* (*34*).

Pharmacology

The symptoms of puffer poisoning in man are well described by Captain James Cook in his journals describing his second voyage around the world, in the *Resolution* (*35*). On 8 September 1774, the ship's clerk procured a fish from the natives of newly discovered New Caledonia. This fish was of a new species, and drawings and a description of it were prepared before it was cooked for dinner. Captain Cook's notes record, "But luckily for us the operation of drawing and describing the fish took so much time till it was too late so that only the liver and roe was dressed of which Mr. Forster [the naturalist of the expedition] and myself did but taste. About three to four o'clock in the morning we were seized with most extraordinary weakness in all our limbs attended with numness of sensation like to that caused by exposing one's hands and feet to a fire after having been pinched much by frost. I had almost lost the sense of feeling nor could I distinguish between light and heavy objects, a quart pot full of water and a feather was the same in my hand. We each took a vomit and after that a sweat which gave great relief. In the morning one of the pigs which had eaten the entrails was found dead. In the morning when the natives came abroad and saw the fish hanging up they immediately gave us to understand it was by no means to be eat, expressing the utmost abhorrance of it and yet no one was observed to do this when it was sold or even after it was bought." In his account of this trip (*36*) Forster describes this fish "as of the genus by Linnaeus named *Tetraodon*, of which several species are reconed poisonous." More modern accounts of tetrodotoxin poisoning in man (*37*) confirm these symptoms and attribute death to respiratory failure.

When the toxin from either puffer fish or newts is injected into mammals it produces muscular weakness and paralysis, fall in blood pressure, and respiratory failure (*5, 12, 13, 37*). In mice, death may occur within 30 seconds after the administration of large doses, even when the toxin is injected subcutaneously rather than intravenously. The first sign of muscular weakness is a wobbling gait in which the hind limbs are slightly splayed, as if the adductor muscles of the thighs were weakened; other muscular groups become paralyzed later. There may be retching and vomiting and clonic convulsions, depending upon the species of mammal and the dose. In fact, tetrodotoxin is probably the most potent known emetic; vomiting occurs after doses of less than 0.3 μg/kg intravenously in dogs (*38*).

Sublethal doses (1 to 5 μg/kg) injected into anesthetized cats, dogs, or rats produce, within a few seconds, a marked fall in blood pressure that is largely the result of vasodilation. At the same time the skeletal muscles begin to show a decrease in response to stimulation of their motor nerves, and eventually they fail to contract when stimulated in this way. Larger doses produce respiratory failure and complete neuromuscular block.

Paralysis of skeletal and smooth muscles, such as the paralysis produced by this toxin, may occur through a direct effect of the toxin on the nerve axon, an effect on autonomic ganglia and neuromuscular junctions, or an effect on the muscles themselves. The toxin in very low concentrations (0.1 to 10 μg/liter) blocks conduction in frog nerves when it is applied directly to the nerve (*12, 39–41*) and blocks neural excitation of the electroplax of *Electrophorus electricus* (*42*). We have eliminated each of the other possible sites as the major point of action. After tarichatoxin had blocked, in cats, contraction of the anterior tibial muscle stimulated through its motor nerve, the muscle still responded to direct electrical stimulation. Intra-arterial injection of acetylcholine at a site near the site of injection of the toxin produced the characteristic contracture-like response, indicating that the motor end plate and muscle fibers were still responsive. Neither the adrenergic receptors in smooth muscle cells of the nictitating membrane and blood vessels nor the myocardium were affected;

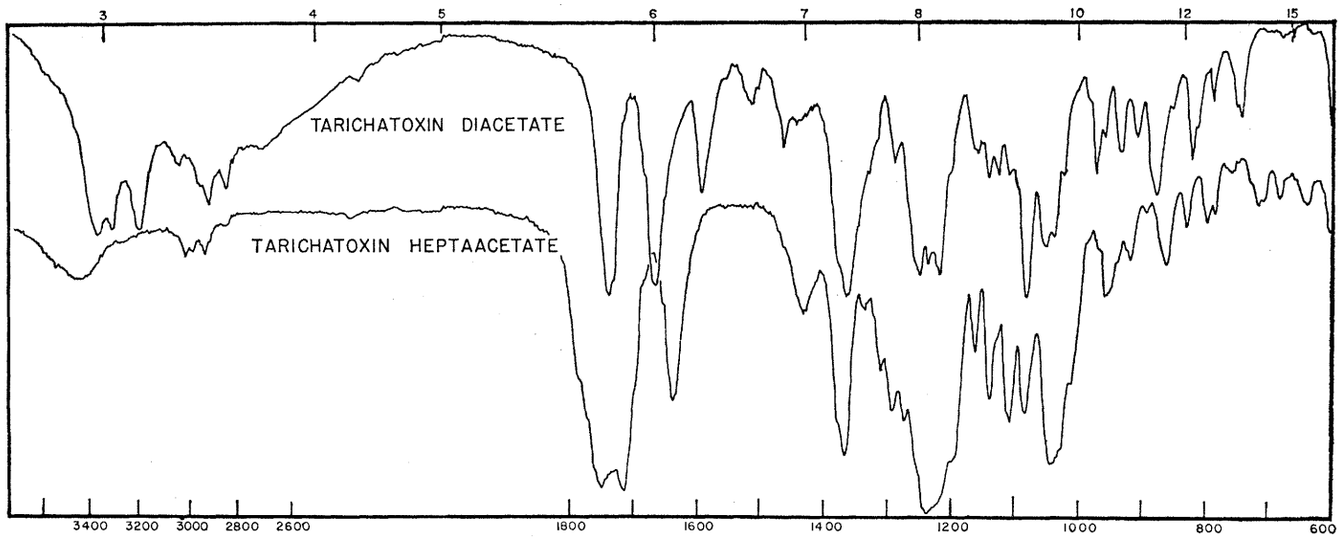


Fig. 8. Infrared spectra of tarichatoxin diacetate and heptaacetate taken in a potassium bromide window. The spectra of the corresponding derivatives from tetrodotoxin were identical to the spectra shown.

they responded to epinephrine and norepinephrine after administration of tarichatoxin. The cholinergic receptors in the autonomic ganglia, the adrenal medulla, and the sinoauricular node were also unaffected by tarichatoxin. They all responded normally to acetylcholine in the presence of the toxin. Thus, it may be concluded that the action of tarichatoxin is primarily on the axons of preganglionic cholinergic and somatic motor nerves (*12*).

In the frog, toxin in higher concentrations blocks direct excitability of skeletal muscle fibers (*40, 43*), decreases the contraction of denervated muscle in response to chemical stimulation (*41*), and blocks the response of the electroplax to direct stimulation (*42*).

In its action, tetrodotoxin resembles local anesthetics more closely than it resembles drugs of any other class. It may be supposed that tetrodotoxin and local anesthetics have basically similar mechanisms of action. Recent experiments have focused on the ability of local anesthetics to prevent the transient increase in permeability to both sodium and potassium ions that normally accompanies the nerve action potential (*44*). Tetrodotoxin appears to differ from procaine and cocaine in acting selectively to prevent or reduce the usual increase in permeability to sodium ion without affecting the permeability to potassium (*45*). In addition, tetrodotoxin is very much more effective than any known local anesthetic. For instance, axonal block is produced by a concentration of about 500 micromoles of cocaine per liter and by a concentration of about 0.003 micromole of the toxin per liter—concentrations differing by a factor of about 160,000.

A simple calculation shows that the effective concentrations of the toxin are approximately the same in vitro and in vivo. In the cat, effects are produced by a dose of 1 μg/kg, or, provided the toxin is distributed uniformly throughout the water of the body (assumed to be 63 percent of the weight), by a concentration of about 1.6 micrograms per liter of fluid. The action potential of frog nerves is blocked by a concentration of about 1 μg/liter in the bathing fluid. Thus, the concentrations required for action in vivo and in vitro are quite similar.

Tetrodotoxin's reputation as a highly potent, quick-acting poison is not confined to the scientific literature. Ian Fleming's *From Russia With Love* ends with the British secret agent James Bond paralyzed and unconscious after a minute wound from a concealed knife. Subsequently, in *Doctor No,* Fleming revealed that the knife blade had been poisoned with tetrodotoxin and that Bond's life had been saved by prompt use of artificial respiration.

Structure

Several empirical formulas for tetrodotoxin have been proposed in the past, but no definitive studies could be made until the toxin had been obtained in a pure state. Tahara (*46*), in 1910, tentatively proposed the formula $C_{16}H_{31}NO_{16}$ for a constituent of "tetrodongifte" which he designated "tetrodotoxin." Now that the toxicity of crystalline tetrodotoxin is known, it appears that his material contained only about 0.2 percent of the active principle. It was not until 1950 that Yokoo (*47*) first reported the isolation of crystalline toxin from the liver and ovaries of *Sphoeroides rubripes.* He called the toxin "spheroidine," and, for the first time, studies of the structure of puffer toxin became meaningful. Yokoo (*48*) proposed the molecular formula $C_{12}H_{17}O_{10}N_3$ on the basis of analysis and cryoscopically determined molecular weight. He later demonstrated (*49*) that "spheroidine" was identical to the material Tsuda and Kawamura (*50*) had isolated from the same source in 1952 and had called "tetrodotoxin," the name originally given the toxin by Tahara. These latter workers favored the formula $C_{12}H_{19}O_9N_3$, as did Kakisawa, Okumura, and Hirata (*51*) for the same toxin, which they isolated from another species, *S. vermicularis,* as well as from *S. rubripes.* Arakawa (*52*) has isolated crystalline tetrodotoxin from still another species, *S. stictonatus,* and it is therefore presumed that this is the toxic principle in all poisonous species of the genus *Sphoeroides.*

On the basis of analysis and the nuclear-magnetic-resonance spectrum of tarichatoxin we proposed the formula $C_{11}H_{17}O_8N_3 \cdot 1/2H_2O$ before we knew that tarichatoxin and tetrodotoxin were identical. The most recent results of x-ray crystallographic studies in the laboratories of Tsuda (*53*) and Nitta (*54*) require this same formula, and

the recent structural proposals of Tsuda *et al.* (*53*) and of Goto, Hirata, and their associates (*55, 56*) are based on this formulation.

The structures of the earlier published degradation products from tetrodotoxin are summarized in Fig. 9. The fact that three of these products (*A, B, C*), formed under acidic as well as basic conditions, possess the quinazoline ring system strongly implicates this sequence for nine of the 11 carbon atoms and all of the three nitrogen atoms in the parent toxin. The possibility cannot be excluded, however, that the toxin has another structural skeleton which rearranges with great facility, under both acidic and basic conditions, to form the stable aromatic quinazoline system. The fourth degradation product (*D*) has not yet been accounted for on any rational basis.

In very recent independent communications, Tsuda's group at the University of Tokyo and at the Sankyo Company (*53*) and Goto and Hirata's group at Nagoya University (*55, 56*) report four new reaction products which contain all of the 11 carbon atoms of the parent toxin. Two of these products (Fig. 10, III and V) contain bromine, and the investigators subjected these two products to analysis by x-ray crystallography, using the heavy-atom technique. Their structures are therefore known with certainty. The same key substance, II, obtained independently by the two groups, was formed through the incorporation of the elements of water after heating of tetrodotoxin in aqueous solution. This substance was called tetrodoic acid by Goto, Hirata, and their associates (*55*) and tetrodonic acid by Tsuda *et al.* (*53*). Treatment of substance II with hydrobromic acid was accompanied by loss of water and the formation of tetrodonic acid hydrobromide (III), whose structure and configuration, as determined by x-ray crystallography, are represented by formula III*a* (Fig. 11). From the x-ray crystallographic determination for substance V (*54*) and the chemical evidence obtained for substances II and IV by Goto, Hirata, and their associates (*55, 56*), it appears that the configuration at C_9, at the end of the oxygen bridge in tetrodonic acid hydrobromide (III*a*), must have been inverted in the process of formation from tetrodotoxin.

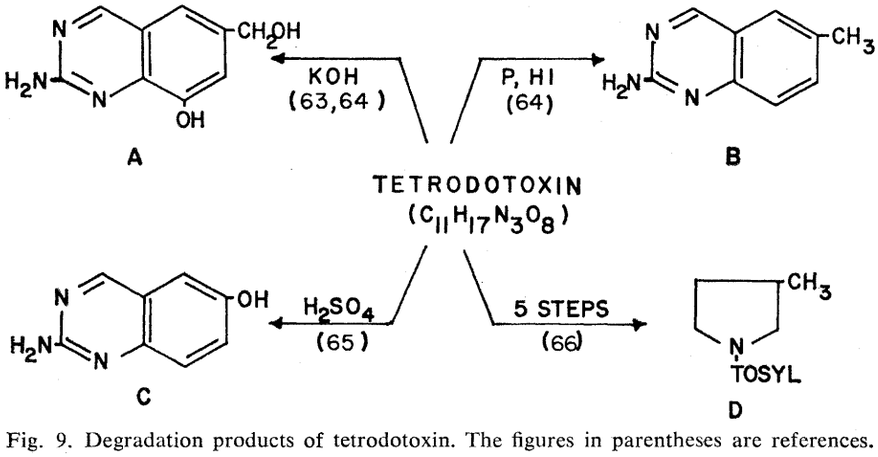


Fig. 9. Degradation products of tetrodotoxin. The figures in parentheses are references.

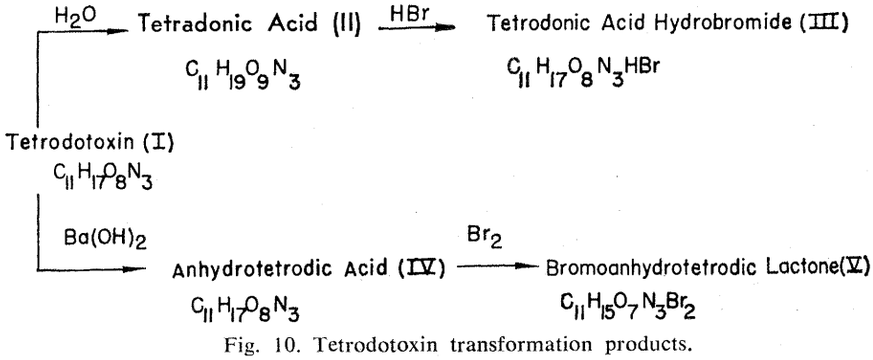


Fig. 10. Tetrodotoxin transformation products.

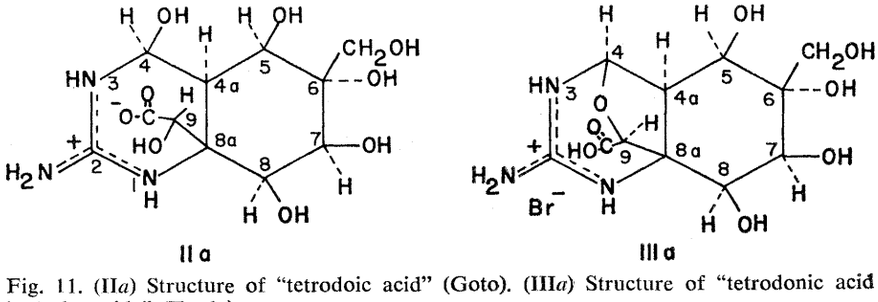


Fig. 11. (II*a*) Structure of "tetrodoic acid" (Goto). (III*a*) Structure of "tetrodonic acid hydrobromide" (Tsuda).

Therefore the problem of the structure of tetrodotoxin is reduced at this time to the determination of that structure (i) which can be converted into tetrodonic acid (II) upon mild treatment with water, and (ii) which is also compatible with all of the properties of the toxin. The most limiting of the chemical and physical properties of tetrodotoxin are (i) its apparent weakly basic nature (pK_a 8.3); (ii) its lack of absorption in the infrared region of 1690 to 2000 waves per centimeter (1690–2000 cm^{-1}); (iii) its lack of absorption maximum above 210 mμ in the ultraviolet; (iv) the 9-cycle-per-second coupling constant between the up-field and down-field protons observed in the nuclear-magnetic-resonance spectrum; and (v) the series of three acetate derivatives with their characteristic nuclear-magnetic-resonance spectra.

The formation of tetrodonic acid by mild treatment of the toxin with water would appear to limit the structure formed through hydrolysis to that of a sensitive lactone, lactam, acetal, hemiacetal, or ortho ester. However, the infrared spectrum eliminates all lactones from consideration. The weakly basic nature of the toxin requires that the guanidine moiety be modified in order that its usual strongly basic nature be masked; the obvious substitution which would produce this modification is acylation.

Goto, Hirata, Nitta, and their associates (*55, 56*) have concluded that I*a* (Fig. 12) represents the structure and I*b* represents the conformation of tetrodotoxin. Tsuda *et al.* (*53*) have

proposed this same structure (with the configuration at C_4 undesignated and that of C_9 inverted) as one of three possible structures for the toxin. Tsuda *et al.*, however, have expressed reservations, believing that the toxin may possibly exist in some dimeric form related to these structures.

Two serious objections can be raised to I*a* as the structure for the toxin. I*a* represents an amide structure (positions 3 and 10) at a bridgehead (position 3). Known examples of such structures are rare, but the most thoroughly studied (*57*), 6,6-dimethylquinuclidone (Fig. 13, VI) has infrared and ultraviolet spectral properties which do not agree with those of the toxin. Resonance (Fig. 13, VII ⟷ VIII), which to a large extent accounts for the stability of amides, cannot operate with nitrogen situated at a bridgehead without violating the injunction of Bredt's rule (*58*) against a double bond at the bridgehead. Woodward (*59*) has discussed this structural problem in connection with penicillin. The infrared stretching frequency of the C=O group in structure VI was reported to be at 5.77 μ, while the toxin absorbs at 6.01 μ. This absorption of the toxin can be attributed to a guanidine group or a normal amide structure but not to a bridgehead amide structure. Furthermore the C=O group in structure VI was reported to have an ultraviolet absorption maximum at 246 mμ (ε 120), unlike that of the pure toxin, which does not absorb significantly in this region.

Another structure which would account for the reduced basicity of the guanidine moiety without formation of a bridgehead amide would be that which derives from tetrodonic acid (Fig. 11, III*a*), by forming an amide bond to the guanidine nitrogen and at the same time cleaving the bond between positions 3 and 4, with formation of a hemiacetal bond to oxygen instead of nitrogen. Tsuda and his associates (*53*) considered two structures of this type, one of which is represented by IX (Fig. 14). The hemiacetal bond in IX is formed between C_4 and C_9. As pointed out by Goto, Hirata, and their associates (*55, 56*), the facility with which the toxin forms the quinazoline derivatives (Fig. 9, *A, B, C*) suggests a structure in which the perhydroquinazoline skeleton is preformed. Furthermore, we are unable to accommodate the nuclear-magnetic-resonance data of the acetates to such a proposal.

Tetrodonic acid hydrobromide (Fig. 11, III*a*) has two hydroxyl groups in a 1,3-diaxial conformation at C_5 and C_7, so oriented that internal ortho ester formation with the carboxyl group at C_{10} in tetrodonic acid (III*a*) is structurally quite possible. Two such ortho ester forms were considered by Goto, Hirata, and their associates (*55, 56*) but were eliminated from serious consideration because the toxin has a pK_a value of 8.3 while normal guanidines have values around 11. This major objection vanishes if the toxin exists as a zwitterion, as represented by structure X (Fig. 14). The pK_a value obtained by titration of an acid solution of such a zwitterion corresponds to that of the weakly acidic group, as in the case of titration of an amino acid hydrochloride. Although little is known about such two-thirds ortho esters which have a free OH group, there is good reason to believe that they would have a pK_a value in the range of 8 to 9. Such a zwitterion structure would also explain the high decomposition point and the low water solubility of the toxin. Finally, this zwitterion structure is the only one to which we have been able to accommodate the nuclear-magnetic-resonance data of the hepta-, penta-, and diacetates (Figs. 6 and 7 (*60*). We have been able to hydrolyze these nontoxic acetates back to the toxin, and therefore we believe they contain the basic toxin structure.

Thus the structural requirements for tetrodotoxin (or tarichatoxin) are rather narrowly defined, and we propose structure X as a solution to the data at hand.

Note added in proof: At the Natural Products Symposium of the International Union of Pure and Applied Chemistry in Kyoto, Japan, 12–19 April, K. Tsuda and associates of the University of Tokyo, T. Goto and associates of Nagoya University, and R. B. Woodward of Harvard University presented

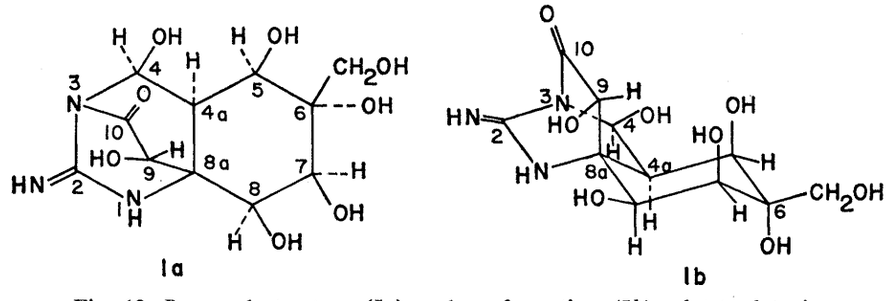


Fig. 12. Proposed structure (I*a*) and conformation (I*b*) of tetrodotoxin.

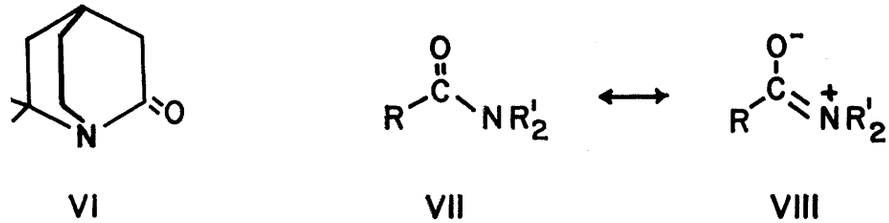


Fig. 13. (VI) Structure of 6,6-dimethylquinuclidone. (VII, VIII) Resonance hybrids of amide structure.

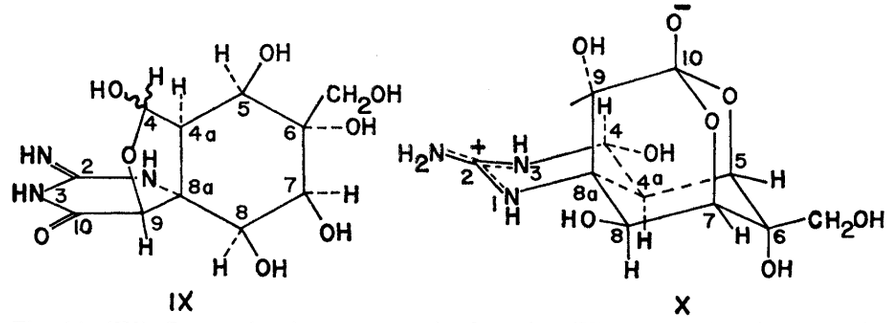


Fig. 14. (IX) Proposed spirane-type toxin formula. (X) Proposed zwitterion-ortho ester formula for tetrodotoxin.

their recent, independent results. There was agreement that in acid solution tetrodotoxin is correctly represented by formula X with a proton neutralizing the negative charge on the zwitterion.

Summary

Tarichatoxin, the neurotoxin first observed by Twitty in the embryos of the California newt *Taricha torosa* in 1934, has been obtained in crystalline form. It has been shown to be identical to tetrodotoxin, isolated from the liver and ovaries of the Japanese puffer fish *Sphoeroides rubripes*. Thus, except in biogenetic studies where the origin of the toxin in amphibians is of importance, the name *tetrodotoxin* should be used for this substance, since this name has historical priority. The toxin has been observed only in amphibians of the family Salamandridae and in fishes of the suborder Tetraodontoidae.

Tetrodotoxin, or tarichatoxin, is one of the most toxic nonprotein substances known and has a mechanism of action resembling that of local anesthetics. However, the minimum detectably effective dose of the toxin is only 1/160,000 the minimum detectably effective dose of cocaine. The chemical structure of tetrodotoxin has been defined recently within narrow limits by two groups of Japanese workers, from classical chemical data and from x-ray crystallographic data on two derivatives. Objections have been raised to the bridgehead amide structure, and an alternate zwitterion–ortho ester structure has been proposed.

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