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Apparent Lack of Tetrodotoxin Biosynthesis in Captured *Taricha torosa* and *Taricha granulosa*

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In order to explore the origin of the potent neurotoxin tetrodotoxin, feeding experiments were carried out using [2-^{14}C]-acetate, [guanido-^{14}C]-arginine, [ureido-^{14}C]-citrulline and [^{14}C(U)]-glucose on the newts *Taricha torosa* and *Taricha granulosa*. The precursors were administered by injection, by oral feeding, or by soaking the newts in a bath of water containing the radioactive precursors. None of these feeding experiments resulted in the incorporation of radioactivity into tetrodotoxin, though common metabolites such as cholesterol, amines, amino acids, and macromolecular compounds were significantly labeled. The toxicities of *T. granulosa* and *T. torosa* used for the experiment were found to be higher than those previously reported. They retained a high level of toxicity during their captivity, yet they were found to continuously release small amounts of toxins (2.5% of the total toxins in one year). When subjected to an electric shock, the newts released significant amounts of toxin.

Keywords——tetrodotoxin; biosynthesis; *Taricha torosa*; *Taricha granulosa*; self-defense mechanism; Californian newt

Tetrodotoxin is one of the most potent neurotoxins known in natural sources.[1,2] Its characteristic actions against the excitable membrane led it to be utilized as an important pharmacological agent in neurophysiology.[3-6] Its structure was established simultaneously by four groups,[1,7] and the total synthesis was accomplished.[8] However, nothing is known about the biogenesis of tetrodotoxin. The animals which are known to contain tetrodotoxin include puffer fishes, gobies,[9,10] Californian newts,[1] gastropods,[11] a starfish,[12] a Costa Rican frog[13] and South Pacific octopus.[14] This apparent lack of a phylogenic relationship among these tetrodotoxin-containing animals, and the seemingly unrelated environments of their habitats make the study of the biogenic origin of tetrodotoxin an extremely intriguing subject. The present work was undertaken to learn whether the endogenous biosynthesis of tetrodotoxin occurs in two closely related species of newts, *Taricha torosa* and *T. granulosa*.

Materials and Methods

Animals and Precursors——*Taricha torosa* were obtained during the regular reproduction period, *i.e.*, from late winter to spring from Prof. D. Wake of the University of California. *T. granulosa* were obtained from Carolina Biological Supply Co., and Mrs. L. N. Nishitani, University of Washington. The newts were kept at 15 °C with the normal light-dark cycle in a glass chamber with pond water. The newts were fed with chips of earthworm every two weeks and the chamber water was changed once or twice a week. The radioactive compounds were obtained from New England Nuclear Co.

Feeding of Precursors and Toxin Separation Procedure——The newts were injected subcutaneously in the inguinal region with the radioactive precursors dissolved in saline (0.65%). In one experiment newts were fed orally with a chip of earthworm impregnated with 2-^{14}C-sodium acetate. The external application of the precursors was done by soaking the newts in 300 ml of pond water containing the radioactive precursors. The pond water and precursors were changed every two weeks. In each experiment the newts were sacrificed either by decapitation or by dipping in liquid nitrogen. The carcasses were cut into pieces and homogenized in methanol (25—30 ml). The

homogenate was adjusted to pH 3.0 by adding acetic acid, and centrifuged. The residue was extracted twice with an equal volume of H_2O–methanol (30 ml), adjusted to pH 3.0 with acetic acid and centrifuged. The extract was evaporated to dryness, suspended in H_2O, and washed with $CHCl_3$ (15 ml × 3). The aqueous extract was then subjected to ultra membrane filtration (Amicon YM-10, molecular weight cut-off of 10000). The low molecular weight fraction was concentrated to a small volume, adjusted to pH 6.6 with dilute NaOH solution, and charged on a column of Bio-Gel P-2 (2.2 × 25 cm). The mixture was eluted with H_2O (200 ml), then with a 0 to 0.1 N acetic acid gradient (300 ml, 5 to 7 ml/8 min), and the radioactivity and toxicity of each fraction were monitored. In some cases the toxin was not well-retained on the Bio-Gel P-2 column. The recovered toxic eluate was then concentrated and applied to an Amberlite IRC-50 (NH_4^+ form, Mallinckrodt Co.) column followed by treatment with activated charcoal.[7c] The mixture extracted from the charcoal was subjected to Bio-Gel P-2 column (2.2 × 25 cm) chromatography, and then the toxicity and radioactivity of each fraction were examined.

Separation of Amines and Amino Acids and Cholesterol——The extraction residues of the newt carcasses were once more extracted as above (H_2O–methanol 30 ml, adjusted to pH 3.0) and washed with $CHCl_3$. The filtrate was then passed through an Amicon YM-10 membrane. The aqueous extract was evaporated to dryness, dissolved in water and filtered. The low molecular weight aqueous extracts were charged on Dowex 50 × 8 columns (0.9 × 7 cm, H^+ form) and the columns were washed with H_2O (15 ml) and 0.1 N HCl (20 ml). The 0.1 N HCl eluate was evaporated to dryness, and then redissolved in 3 ml of H_2O. The total amounts of amines and amino acids were estimated by the fluorometric method,[15] using 0.5 ml of each sample with 1.5 ml of 0.2 M boric acid, which had been preadjusted to pH 9.0 with NaOH solution, and 0.5 ml of 0.03% fluorescamine in acetone. The solutions were measured for fluorescence with excitation at 390 nm and emission at 490 nm, using blank and standard solutions of 10 and 100 mmol isoleucine. The newt carcass residues were also extracted with methanol (20 ml) and a methanol–$CHCl_3$ mixture (1 : 1, 20 ml). The extracts were evaporated to dryness, dissolved in 3 ml of 10% NaOH in 80% ethanol and refluxed for 2 h. After being diluted with H_2O, the mixture was extracted with ether (5 ml × 2). The ether extracts were evaporated to dryness, dissolved in small volumes of $CHCl_3$ and then subjected to preparative TLC (each 10 × 20 cm, Silica gel HF, Analtech Uniplate). The sterol bands were detected by spraying H_2O and extracted with ethyl acetate after drying the plate. Evaporation of the solvent gave crystalline sterols whose radioactivity was counted.

Bioassay and Thin-Layer Chromatography (TLC)——The mouse assay was carried out by injecting test solutions intraperitoneally into mice and the toxicity was expressed in terms of mouse units (MU) using the death time-toxicity curve given by Hashimoto and Migita.[16] When given by intraperitoneal injection, one mg of purified tetrodotoxin corresponds to a toxicity of 6250 MU.[17] Thin-layer chromatography of the toxin mixture was performed on silica gel plates (Whatmen LHP-K) using the solvent system pyridine–ethyl acetate–H_2O–acetic acid (15 : 10 : 4 : 3). Plates were sprayed with 10% KOH in methanol, heated at 120—130 °C for 5 min and viewed under a 366 nm UV lamp. Riboflavin and other fluorescent compounds were examined on TLC by using the solvent system acetone–acetic acid–methanol–benzene (1 : 1 : 4 : 14), and detected under a 366 nm UV lamp.

Electric Shock Treatment of Newts——Electric shock was applied to the newts by dipping the newts for 10 to 20 s in pond water (250 ml) in an electrophoresis chamber running at 400 V and 16—23 mA. The released toxin was analyzed by mouse assay during the subsequent 24 h.

Results

Toxicities of *T. torosa* and *T. granulosa*

Examination of ten *T. torosa* showed toxin contents ranging from 3760 to 6000 MU per animal, the average toxicity being 4970 MU (*ca.* 0.7 mg of tetrodotoxin). The males and females seemed to be equally toxic in this case. In *T. granulosa*, the individual variation was greater. The least toxic animal contained 500 MU while the highest value was 61500 MU from the combined extract of 3 newts. The average toxicity for 273 animals was 10800 MU (*ca.* 1.5 mg of tetrodotoxin). One group of *T. granulosa* (179 newts with an average body weight of 9.2 g) had an average toxicity of 14000 MU or *ca.* 2 mg of tetrodotoxin per animal. After 167 d of captivity the average body weight of the newts had decreased to 7.63 g. The toxicity decreased accordingly to 10800 MU, but the toxicity per gram body weight remained almost unchanged.

Feeding of [2-^{14}C]-Acetate, [ureido-^{14}C]-Citrulline, [guanido-^{14}C]-Arginine, and [^{14}C(U)]-Glucose

In the experiment in which [2-^{14}C]-acetate was given to newts by injection and kept for 6 and 13 d (Fig. 1), or given orally for 13 d, significant labelings of aqueous extracts were observed in both high molecular weight (>10000) and low molecular weight (<10000)

fractions (Table I). In the Bio-Gel P-2 column chromatography of the low molecular weight fractions, most of the radioactive compounds were eluted with H_2O (Fig. 1). Tetrodotoxin, which was eluted with dilute acetic acid, showed no detectable radioactivity in any of these experiments. In the newts which were kept for 4 weeks in water containing 2-^{14}C-acetate, the incorporation of radioactivity in the toxic fraction was also nil. A radioactive peak, which was found in the fractions just prior to the tetrodotoxin fraction, was clearly separated from the toxin in the second chromatography. The nature of the responsible compound was not identified. This fraction increased when the soaking period was extended to 86 d and was also found in the feeding experiments with radioactive arginine (Fig. 1) and citrulline.

No incorporation of the radioactivity into tetrodotoxin was observed in the feeding of [ureido-^{14}C]-citrulline, [guanido-^{14}C]-arginine or [^{14}C(U)]-glucose. External feeding of [^{14}C]-citrulline was attempted by soaking the newts for 4 weeks in water containing [^{14}C]-citrulline. Again, no incorporation of radioactivity into tetrodotoxin was observed.

In contrast to the total lack of incorporation into tetrodotoxin, sterols (mostly cholesterol), free amines, amino acids and macromolecular compounds were found to be significantly labeled in all the feeding experiments. Thus, the administered precursors were indeed absorbed into the newt body, and utilized in the metabolic system. The results of all the feeding experiments are summarized in Table I.

Examination of the Newt Chamber Water and Electric Shock Treatment of Newts

Examination of the newt chamber water revealed that the newts were releasing the toxin at a rate of 12.6 MU per newt over a two-week period. When shocked by passing a 16—23 mA current at 400 volts through the water, a newt suffered syncope and released 1360 MU of toxin during 24 h. When the recovered newt was shocked 3 times during a 2 week period, it released 635, 1100, and 2830 MU of toxin in the subsequent 24 h periods, though the newt died 1 d after the last electric shock. At the time of the third electric shock, the amount of toxin released by the newt during the first 30 min was 285 MU in 30 s, 830 MU in 2 min, and 1160 MU in 30 min. The amount of the toxin released from the shocked newt was 5940 MU, including that released after death. The residual toxin extracted from the carcass was 5700 MU. Thus, the total toxicity (11640 MU) was close to the average toxicity (10800 MU).

Following their deaths in captivity, the newts were observed to exude significant amounts of riboflavin into the bath water. In live newts, the flesh all over the body is rich in riboflavin.

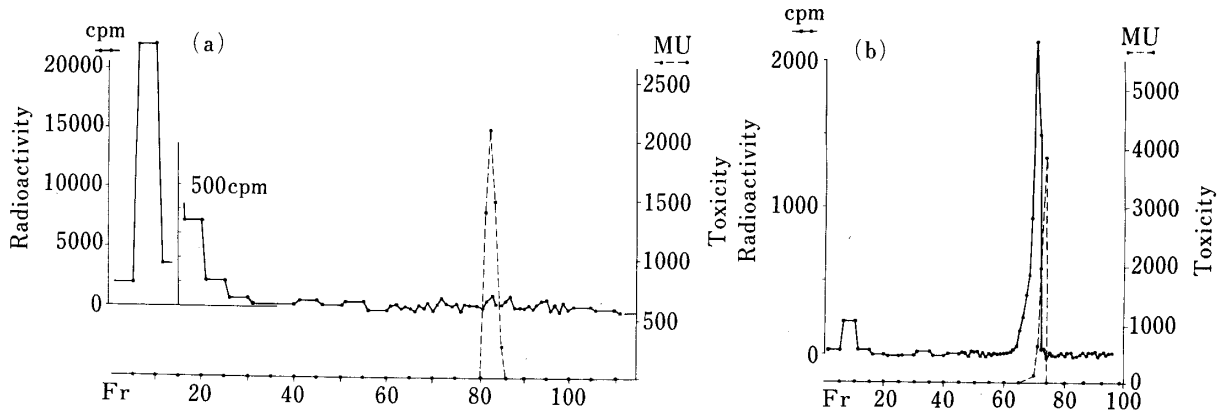

Fig. 1. Toxicity and Radioactivity Patterns of the Toxic Fractions from *T. torosa* Fed with 9.44 μCi of [2-^{14}C]-Acetate and Kept for 13 d (**a**) and from *T. granulosa* Fed with 10 μCi of [guanido-^{14}C]-Arginine and Kept for 50 d (**b**)

Fractions (6.6 ml each) were collected from a Bio-Gel P-2 column (2.2 × 25 cm) with the use of acetic acid gradients. The toxin fraction in (**b**) was chromatographed after IRC-50 chromatography followed by treatment with activated charcoal (see the text).

TABLE I. Radioactivities in the Fractions from Newts Fed with Labeled Precursors

Precursor	No. of newts	Species[a] and feeding method	Precursor (μCi) and feeding period (d)	Total radioactivity (cpm) in water soluble fraction		Radioactivity (cpm/mmol) in cholesterol	Radioactivity (cpm/mmol) in amines and amino acids	Radioactivity in tetrodotoxin
				M.wt. > 10000	M.wt. < 10000			
[2-^{14}C]-Acetate (35.9 mCi/mmol)	2	T.t (Injection)	9.44 (6)	51000	293000	172000	1.7×10^5	0
	2	T.t (Oral feeding)	9.44 (13)	35900	190000	34300	8.9×10^5	0
	2	T.t (Injection)	9.44 (13)	83500	185000	29600	1.1×10^5	0
	1	T.g (Soaking)	9.44 (28)	7400	24400	16500	4.5×10^6	0
	2	T.g (Soaking)	51.92 (86)	25000	67500	29500	1.5×10^7	0
[ureido-^{14}C]-Citrulline (53 mCi/mmol)	2	T.t (Injection)	10 (18)	69200	1128000	28400	6.3×10^6	0
	1	T.t (Injection)	5 (86)			3200	2.6×10^6	0
	1	T.t (Injection)	5 (105)	78500	67800	1700	8.2×10^5	0
	1	T.g (Soaking)	20 (33)	17500	64500	6100	1.1×10^5	0
[guanido-^{14}C]-Arginine (23.0 mCi/mmol)	2	T.g (Injection)	10 (50)	92600	180000			0
[^{14}C(U)]-D-Glucose (2.2 mCi/mmol)	3	T.g (Injection)	15 (26)	558000	4827000			0

a) T.t = *Taricha torosa*, T.g = *Taricha granulosa*.

However, no riboflavin is found in the intestine or skin. The dead newts, however, showed intense fluorescence due to the exuded riboflavin in the skin of the abdomen, jaw and eyes when viewed under a 366 nm lamp.

Discussion

The toxicity of *T. granulosa* used in the present study was remarkably higher than that reported by Wakely *et al.*[18] The amount of tetrodotoxin in *T. Granulosa* was reported to range from 2 to 36 μg per gram of newt, corresponding to 125—2250 MU for an average body weight of 10 g. The toxicity of *T. granulosa* in the present study was 8 to 328 μg or 500 to 20500 MU per ten gram newt. Both male and female animals were found to be toxic.

Although on one occasion a polyketide origin for tetrodotoxin was suggested,[7b] it is difficult to envision a structural feature clearly identifiable with a known category of natural products. In other words, the complex tetrodotoxin molecule could arise from any of the common precursors, such as isoprenoids, shikimates, sugars, polyketides, *etc.*, except for the guanidine moiety, which may very possibly be derived from the urea cycle. Our choice of precursors used in this experiment was made with this consideration in mind. We found significant incorporation of these selected precursors into such common metabolites as cholesterol, amines, amino acids, and macromolecular fractions, indicating that they are reasonably well metabolized in the newt body.

The fact that there was no incorporation of the precursors into tetrodotoxin could be due to various reasons. One distinct possibility is that the toxin is biosynthesized in the gastrointestinal tract or on the skin. However, the precursors given orally and externally were not incorporated into the toxin while other metabolites were reasonably labeled in both experiments. This apparent lack of biosynthesis of tetrodotoxin in the newts can be interpreted in several ways: 1) tetrodotoxin is synthesized only during a very limited period of the developmental stage and its turnover is extremely slow, 2) tetrodotoxin is synthesized only under certain stressed conditions as a self-defense mechanism, 3) tetrodotoxin or a key precursor comes from a special dietary source, 4) tetrodotoxin is produced by a symbiotic microorganism, but in captivity, the conditions are not suitable for the organisms to grow or to produce the toxin, 5) the precursors cannot reach the toxin synthesis site when given by the methods employed in the present study.

The failure to label tetrodotoxin using labeled acetate, citrulline, arginine and glucose in the present study and the poor incorporation of cholesterol into batrachotoxin (*vide infra*) seem to indicate that while these compounds cannot necessarily be ruled out as toxin precursors, they at least have great difficulty in reaching the actual toxin synthesis site under the conditions of methods employed. Such a difficulty also occurred in the feeding of many steroidal precursors to toads, which resulted in rather poor incorporation of precursors into bufadienolides.[19,20]

Tetrodotoxin contents in puffer fish and newts were reported to be high in the reproductive period. This fact is taken to be an indication of the *de novo* synthesis of the toxin in the animals. However, this may not necessarily be the case, since many symbionts, *e.g.*, protozoa in amphibians, are known to have a life cycle closely related to the hormonal changes in the host.[21] Nevertheless, we tried to coincide the feeding experiments with the reproduction period, with no success. We observed little seasonal variation in the toxicity, and the animals used for the experiments maintained a very high toxicity (*ca.* 0.2 mg/g body weight) which they might already have had for some length of time. It is noteworthy that the newts retained a high level of toxicity and they released the toxin in small amounts (12.6 MU in two weeks). This is equivalent to 0.1% of the total toxin in the newt. If we consider the degradation of the toxin in water, this value might be even higher. This means that

during a whole year at least 2.5% toxin turnover should occur. Accordingly, in spite of the failure of the labeling experiments in the present study, it is still possible that the newts are synthesizing tetrodotoxin during captivity.

Stress seems to increase the release of the toxin implicating its use as a self-defense mechanism. When subjected to a strong electric shock, the newts released a large amount of toxin. Shocking newts 4 times during 2 weeks caused the newts to release toxin which is nearly equivalent to 1 mg of tetrodotoxin per animal. The total toxicity (11640 MU) of the released toxin and the toxin remaining in the body matched the average toxicity of the newts used in the present study. This suggests that rapid replenishment of the toxin does not occur after the release of the toxin. The newts were found to contain large amounts of riboflavin. Whether riboflavin is related to the toxin production remains uncertain.

Significantly, our results have a remarkable resemblance to those observed in a biosynthetic study of another potent amphibian neurotoxin, batrachotoxin. Feeding of [2-^{14}C]-acetate to the Colombian frog, *Phyllobates aurotaenia*, gave no significant incorporation into the toxin, while a high incorporation was observed into cholesterol.[22] Cholesterol, the most plausible precursor of steroid derivatives, was also found to be a poor precursor to batrachotoxin in the frog. The frogs slowly lose the toxin but still retain a high toxin level for as long as six years of captivity. In spite of the failure of the labeling experiments, the prolonged retention of batrachotoxin in the frogs suggested that the frogs continue to synthesize the toxin in captivity.[23] A remarkable fact is that the F_1 frogs reared in captivity were completely devoid of the toxin.

The levels of tetrodotoxin in the puffer fish *Fugu riphobles* are known to vary extensively according to the locality.[24] A more intriguing fact is that when innocuous puffers were fed with a diet containing pure tetrodotoxin, almost no accumulation of the toxin was observed in the tissues, while the fish fed with the toxic ovaries of puffer fish became substantially toxic.[25] Clearly the indication is that the neurotoxins tetrodotoxin and batrachotoxin are formed through a unique biogenetic mechanism not observed with other ordinary secondary metabolites. This, along with the capricious occurrence of the uniquely structured toxin in such distantly related animals, strongly hints that there is a key factor missing in the whole picture. For example, it is possible that tetrodotoxin is distributed in many terrestrial and aquatic vertebrates and invertebrates either as a derivative or as a precursor which is not toxic, and in a few species, under the influence of environment or a symbiotic organism, it is transformed into the intensely toxic compound.

At present, our knowledge about the origin of toxic compounds in marine organisms is very limited. Nevertheless, we see growing evidence that animals receive functionally important compounds from other organisms either through the food chain or symbiosis. The self-defensive allelochemical substances in marine invertebrates or bioluminescent substances in fishes are good examples. Amazingly, many fished utilize luciferins derived from the diet or maintain bioluminescent bacteria in the luminescent organs.[26] A similar situation may exist in the biogenesis of tetrodotoxin and other marine toxins.

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