changes. Since colonies of rodents tend to feed and drink at sites which adults of the colony frequent, we suggest that weanling mice "learn" to drink the solution consumed by their adult models. Alcohol intake data in these strains have been interpreted (*14*) to indicate the complex inheritance of the alcohol preference; it seems not unlikely that social pressures might operate to affect the reinforcing value of the taste of alcohol, a factor given first consideration by Fuller and Collins (*14*).

Whether critical periods exist for environmental influences to be effective in mice, as in man, is not known. Whether adult subjects with established drinking behaviors would be influenced as readily, and as substantially, as the young mice in this experiment, or whether the alteration in drinking behavior we have observed persists beyond our test period can only be answered by further experiments. The extrapolation to man of the data of such further experiments may, of course, never be warranted.

CARRIE L. RANDALL*, DAVID LESTER
Center of Alcohol Studies,
Rutgers University,
New Brunswick, New Jersey 08903

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9. The genetically based predisposition for an alcohol solution receives further support from the results of ova transfer experiments carried out in our laboratory [*Nature (Lond.)* **255**, 147 (1975)]. The strain-typical preference or aversion for an alcohol solution on the part of C57BL or DBA mice remains substantially unaltered when fertilized ova are exchanged between the strains and the offsprings' self-selection is subsequently tested, emphasizing the major role of heredity and the lesser, though significant, role of maternal and environmental manipulations.
10. Some male adult mice, especially those of the C57BL strain, killed weanlings, leading to the unequal N of Table 1. Thus, a total of 13 weanlings of both strains were housed with adult females, rather than with males; no difference in alcohol consumption was apparent between these subjects and those housed with male adults.
11. Since male DBA mice were not significantly different from female mice in their fluid intake, the data of both sexes were pooled and treated together. There were too few ($N = 4$) female C57BL mice to be used alone, and since two of the three control female mice differed from the male control C57BL mice in their alcohol intake, the data from female C57BL mice were not used.
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* C.L.R. was supported in part by a predoctoral fellowship from the Charles and Johanna Busch Memorial Fund of Rutgers University; in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Neuroscience, School of Medicine, University of Florida, Gainesville; postdoctoral fellow of the National Institute on Alcohol Abuse and Alcoholism.

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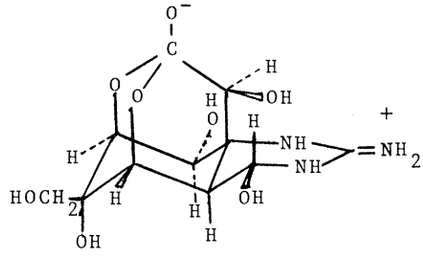
Tetrodotoxin: Occurrence in Atelopid Frogs of Costa Rica

Abstract. *The potent neurotoxin tetrodotoxin, which has previously been found in puffer fish of the order Tetraordontiformes, a goby (*Gobius criniger*), and the California newt (*Taricha torosa*), has now been identified in the skins of frogs of the genus* Atelopus *from Costa Rica.*

Tetrodotoxin (*1*), the structurally unique and pharmacologically potent neurotoxin, was first isolated from Japanese puffer fish (*2*), primarily *Spheroides rubripes*, and subsequently from the California newt, *Taricha torosa* (*3*), and other members of the genus *Taricha* (*4*). The occurrence of tetrodotoxin in two such different animals as the newt and the puffer fish was surprising in view of the fact that this compound does not appear to be a substance readily accessible via the known biogenic pathways from acetate, mevalonate, or the amino acids (*5*). More recently, tetrodotoxin has been isolated from a completely different fish, a goby (*Gobius criniger*) from Taiwan and Amami-Oshima Island (*6*). We now report that we have identified tetrodotoxin in extracts of skin from three populations of frogs of the genus *Atelopus* from Central America (*7*). We have found that aqueous extracts of skin of approximately 12 different species, subspecies or distinct populations of atelopid frogs, so far tested by us are toxic when injected intraperitoneally into mice. In four of these (Table 1) we were able to obtain enough material so that some information concerning the chemical nature of the toxin could be deduced. This was done by techniques previously described (*8*), combined with final purification with gel filtration chromatography (pyridine acetate buffer, at 5°C, on Bio-Gel P-2). Concentrates with an activity in the order of 4000 to 6000 mouse units per milligram (*9*) were obtained. The toxins were found in the skin of both males and females, but not in the viscera, muscle, or bone. In one group of male frogs it was found that the toxin was uniformly distributed in the skin dorsally, ventrally, and along the limbs. Solid tetrodotoxin ultimately separated from highly purified aqueous concentrates of the skin of *A. varius ambulatorius* (Table 1). It was identified by use of proton nuclear magnetic resonance (NMR) spectroscopy [Varian XL-100 NMR instrument in Fourier transform mode (*10*)] by direct comparison with a genuine sample. Tetrodotoxin has a unique NMR spectrum with a slightly broadened upfield doublet coupled with a sharp downfield doublet (D_2O plus CD_3COOD as solvent; δ 2.76, 5.91 ppm; J = 9.5 hz, 1H). Both signals appear in a region unencumbered by other signals and are readily recognized. Furthermore, the mass spectra of tetrodotoxin from these frogs and from puffer fish are identical (*11*). In addition, tetrodotoxin was the major and perhaps only toxic constituent in the concentrates from skin of *A. varius varius* (Table 1).



Tetrodotoxin, $C_{11}H_{17}N_3O_8$

From a population of frogs identified by Savage (*12*) as *A. chiriquiensis* (Table 1), we obtained a skin extract that proved to be a mixture of approximately 30 percent tetrodotoxin and a second major component which we have designated chiriquitoxin after the species in which it is found. After separation from tetrodotoxin by liquid-liquid chromatography (250 pounds per square inch, Bio-Gel P-2, pyridine-acetate buffer) it is clearly distinguishable from it by its NMR spectrum. The chiriquitoxin spectrum bears a certain resemblance to that of tetrodotoxin, especially with respect to the coupled upfield-downfield doublets.

We have also reexamined the previously obtained skin extracts from the Panamanian frog *A. zeteki* (*8, 9*) (Table 1) from which we had isolated a substance that had been designated atelopidtoxin and is now called zetekitoxin. We have been unable to detect either tetrodotoxin or chiriquitoxin in the *A. zeteki* extracts. We are confident that we could easily detect,

Table 1. Toxicity and occurrence of tetrodotoxin in Costa Rican frogs.

Frog					Tetrodotoxin	
Species	Number	Dorsal color	Origin	Average weight (g)	Toxicity per frog (M.U.)*	Percent of total
a. *Atelopus varius (A. varius varius)*	375	Black and red on yellow	San Antonio de Patarra, San Jose province, Costa Rica	1.8	100	~100
b. *Atelopus varius (A. varius ambulatorius)*	99	Mottled black on green	Valle de Parrazu, Costa Rica, also San Carlos, N. Costa Rica	1.3	120	~100
c. *Atelopus chiriquiensis*	93	Uniform yellow, yellow-green, grey, or rust-grey	Cerro de la Muerte, S. Costa Rica	1.6	350	~30
d. *Atelopus varius (A. varius zeteki)*†		Black on orange or orange	El Valle de Anton, Panama	6.2	1200	>5

*M.U., mouse units; 1 M.U. is the amount of toxin necessary to kill a 20-g mouse within 1 hour when the toxin is administered intraperitoneally in a volume of 0.2 ml. †See (*8, 9*).

by the NMR method being used, 5 percent of tetrodotoxin or chiriquitoxin (or both). Probably half this amount would be observed.

Savage (*12*) has published a systematic revision of the genus *Atelopus* of Costa Rica and Panama in which he recognized only three species in this region: *A. varius, A. chiriquiensis*, and *A. senex*. Several other populations are classified by Savage as *Atelopus varius*. Earlier investigators recognized these as distinct species or subspecies on the basis of their distinctive patterns of coloration. It is thus not surprising that we found that tetrodotoxin was present in the skin of both populations listed as (a) and (b) in Table 1 (those populations previously designated as *A. varius varius* and *A. varius ambulatorious*). On the basis of NMR spectra, we are certain that zetekitoxin (*8, 9*) is distinct from both tetrodotoxin and chiriquitoxin. In addition, the pharmacological action of zetekitoxin is different from that of tetrodotoxin. Some studies on the new chiriquitoxin show that it also is distinct from zetekitoxin but that it closely resembles tetrodotoxin in pharmacological action.

The occurrence of tetrodotoxin in widely different animals (puffer fish, a goby, newts, and frogs) suggest that this toxin may have some physiological function in animals aside from its possible one of affording protection from predators. Heilbrunn *et al.* (*13*) suggested that tetrodotoxin may have antimitotic properties similar to those of substances extracted from ovaries of starfish. However, in the experiments made by Couillard (*14*) in Heilbrunn's laboratory, the antimitotic substance extracted from the spent ovaries of Atlantic puffers, which in any case contain very little tetrodotoxin (*15*), had chemical properties quite different from those of tetrodotoxin. In our laboratory (*16*) we found that tetrodotoxin in concentrations as high as 1 mg/liter had no effect on the growth of mouse fibroblasts in cell culture (measured by counting and by protein analysis) over a 4-day period. Habermehl and Preusser (*17*) have proposed that the polypeptide toxins found in the skin of *Leptodactylus* serve as antimicrobial or antifungal agents. We could not demonstrate any antibacterial effect of tetrodotoxin on *Staphylococcus aureus, S. albus*, or *Streptococcus pyrogenes* (*18*). In addition we have observed unidentified bacteria and fungi growing in concentrated solutions of the toxin from *A. zeteki* as well as *A. varius varius*. For this reason, we purified our extracts at 5°C and always passed them through bacterial filters. Thus if tetrodotoxin has any physiological function in the various animals in which it occurs, it remains to be found.

The erratic distribution of tetrodotoxin in *Atelopus* and *Gobius* (*6*) may suggest again the possibility that it originates in the food of these animals (*19*). However, it seems quite unlikely that a widely distributed toxic plant or microorganism that could serve as a food source (either directly or indirectly) for fish, newts, and frogs would have eluded detection until now. It seems more logical to assume that the ability to synthesize tetrodotoxin was a coincidental genetic development in certain fishes and amphibians either because it has survival value or because it is a metabolic end product that happens to be toxic.

The finding of three distinct very potent toxins (tetrodotoxin, zetekitoxin from *A. zeteki* (*8, 9*), and chiriquitoxin, the new tetrodotoxin-like substance from *A. chriquiensis*) among the closely related *Atelopus* of Costa Rica and Panama is of great significance for any attempt at biochemical taxonomy of this genus. The identification of the specific toxins responsible for the toxicity found in this group of frogs and their distribution among the other *Atelopus* of Central and South America remain to be determined.

YONG H. KIM
GEORGE B. BROWN
HARRY S. MOSHER
Department of Chemistry, Stanford University, Stanford, California 94305
FREDERICK A. FUHRMAN
Department of Physiology, Stanford University, and *Hopkins Marine Station of Stanford University, Pacific Grove, California 93950*

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