Toxin from Skin of Frogs of the Genus Atelopus: Differentiation from Dendrobatid Toxins

Abstract. *A potent, dialyzable toxin (atelopidtoxin) occurs in the skin of frogs of the genus* Atelopus. *A concentrate of atelopidtoxin from* Atelopus zeteki *has an* LD_{50} *in mice of 16 micrograms per kilogram. It differs from batrachotoxin, tetrodotoxin, and saxitoxin, the only known nonprotein substances of greater toxicity, as well as from all toxins previously isolated from amphibia.*

Certain vividly colored frogs from Central and South America have been known for centuries to be highly poisonous. The jungle tribes knew well that potent arrow poison could be made from their skins (*1*). Witkop, Daly, and their associates (*2–4*) isolated two groups of toxins from the skins of such frogs belonging to the family Dendrobatidae, and characterized the toxins chemically and pharmacologically. We report here the partial purification and pharmacological properties of a new toxin from a different family of brightly colored frogs, the Atelopodidae, that also are found in Central and South America. This toxin, atelopidtoxin, differs chemically and pharmacologically from the dendrobatid toxins.

Dunn (*5*) wrote that in El Valle de Anton of northwest Panama the golden arrow frog, *Atelopus zeteki*, is strictly diurnal and that the brilliant orange-yellow of its skin contrasts sharply with the black rocks on which it rests (Fig. 1). If color serves as a warning to predators, as has been suggested, these frogs should be poisonous. But we have found no record in the literature of any experimental work on the chemical or pharmacological properties of a toxin from them, or, indeed, of evidence of their toxicity (*6*).

During the last 4 years we have obtained several shipments of *A. zeteki* from commercial sources. Dialyzates of aqueous extracts of either whole frogs or frog skins were tested for toxicity by intraperitoneal injection of volumes of 0.1 to 0.4 ml into white mice. The extracts were found to be highly toxic. One mouse unit is defined as the amount of toxin required to kill a 20-g mouse.

After lethal doses the first sign of intoxication is a wobbling of the hind legs. This may occur within 1 minute, and with large doses death occurs as early as 2 minutes after injection. With smaller doses death may be delayed for 20 to 30 minutes, but mice surviving any longer survive indefinitely. Death is preceded by clonic convulsions. When the respiration ceases, immediate opening of the thorax shows no signs of contraction of the heart.

More highly concentrated extracts of the toxin were obtained from the skin by several methods. For example, in a typical experiment, the skins from four *A. zeteki* (2.02 g) were minced finely with scissors and homogenized with five volumes of distilled water in a Waring Blendor. The homogenate was placed in Visking dialysis tubing and dialyzed three times for 12 hours each at 4°C against an approximately equal volume of distilled water in a rocking, rotating dialysis apparatus. The dialyzates were combined and evaporated to dryness by lyophilization. About 1800 mouse units of toxin were recovered. The dry powder was extracted twice with 5-ml portions of chloroform, and the chloroform was discarded. The residue was then extracted twice with 5-ml portions of methanol, and the methanol was discarded. The dry residue was dissolved in 2 ml of distilled water and centrifuged, and the supernatant was decanted. The residue was reextracted once with 2 ml of distilled water, and the supernatant fluid was combined with the first extract. The combined extracts (3.75 ml) contained about 1700 mouse units of toxin. Acetone was added to give a final concentration of 58 percent by volume and the mixture was centrifuged. The precipitate consisted of a lower gray solid (not toxic) and an upper brownish syrup that was highly toxic. This was carefully removed with a fine-tipped pipet and was found to contain about 1300 mouse units of toxin, or a yield of about 70 percent. This material was used for the preliminary pharmacological experiments. This toxin, which we shall designate atelopidtoxin, has been obtained in a more highly purified form by a modification of the above procedure followed by gel filtration, column chromatography on cellulose, and precipitation from water with acetone as the final step (*7*). The LD_{50} of the most active preparation is about 16 μg/kg by intraperitoneal injection into mice. Intravenous injection of doses of 3 to 8 μg of atelopidtoxin per kilogram into anesthetized rabbits or dogs results in hypotension followed by various bizarre cardiac rhythms, atrioventricular block, and terminal ventricular fibrillation (*8*). In frogs (*Rana catesbeiana*), intravenous administration of the toxin produces atrioventricular block with stoppage of the ventricle in diastole. But of great interest to cardiologists is the ability of the toxin to produce "circus movement" contraction of the atria, or ventricle, or both (see Rytand, *9*). Evidence of this striking phenomenon has been obtained by cinematography (*10*).

Fig. 1. *Atelopus zeteki.* Frogs are bright orange-yellow or orange-yellow with various patterns of black bands on the dorsum. They may be up to 6 cm in body length and have an average body weight of 5 to 6 g.

The skin of a single *A. zeteki* contains about 500 mouse units of toxin. Aqueous extracts of the skins of four other species of *Atelopus* from Costa Rica and Colombia have also been prepared as described above and assayed by injection into mice. The skins of *A. varius varius, A. varius ambulatorius,* and *A. cruciger* contain 30 to 150 mouse units per skin of a toxin that produces symptoms in mice identical to those produced by atelopidtoxin. The skin of *A. planispina* contains about 10 mouse units per skin of a toxin that produces a different sequence of symptoms when injected into mice. These include marked depression from which the mouse can be aroused, and finally cessation of respiration before the heart stops beating.

Our data demonstrate that the toxin from the skin of *A. zeteki* is clearly different from known toxins found in related poisonous amphibia. The high toxicity alone differentiates it from both toxins from *Dendrobates pumilio* (*4*), from bufotoxin or any of the other steroidal derivatives from the genus *Bufo* (*11*), from samanderine (*12*), or

from any of the known catechol, indole, or imidazole alkylamines (*13*). Of known nonprotein toxins, only batrachotoxin (*2*), tetrodotoxin (*14*), and saxitoxin (*15*) are more toxic than the concentrate from *A. zeteki* which has been prepared in our laboratory (*7*). Atelopidtoxin can be differentiated chemically from batrachotoxin, which is a steroidal alkaloid readily soluble in chloroform and other organic solvents (*2*). The insolubility of atelopidtoxin in chloroform is indicated by our procedure for extraction. Furthermore, in separate experiments we have been unable to extract atelopidtoxin with chloroform from aqueous solutions at *p*H 2, 8, or 11. Thus, the markedly different solubilities of atelopidtoxin and batrachotoxin demonstrate that they are not identical. Atelopidtoxin differs in pharmacological action from both tetrodotoxin and saxitoxin, which block axonal conduction without significant cardiotoxic effect. In addition, atelopidtoxin has been differentiated from tetrodotoxin and saxitoxin by direct comparison using thin-layer chromatography (*7*).

FREDERICK A. FUHRMAN
GERALDINE J. FUHRMAN
Fleischmann Laboratories of the Medical Sciences, School of Medicine, Stanford University, Stanford, California 94305
HARRY S. MOSHER
Department of Chemistry, Stanford University

References and Notes

1. L. Lewin, *Die Pfeilgifte* (Barth, Leipzig, 1923).
2. F. Märki and B. Witkop, *Experientia* **19**, 329 (1963); J. W. Daly, B. Witkop, P. Bommer, K. Biemann, *J. Amer. Chem. Soc.* **87**, 124 (1965).
3. J. W. Daly and B. Witkop, *Mem. Inst. Butantan* **33**, 425 (1966); T. Tokuyama, J. Daly, B. Witkop, I. L. Karle, J. Karle, *J. Amer. Chem. Soc.* **90**, 1917 (1968).
4. J. W. Daly and C. W. Myers, *Science* **156**, 970, 1967.
5. E. R. Dunn, *Occas. Pap. Boston Soc. Natur. Hist.* **8**, 65 (1933).
6. Another atelopid, *Atelopus* (*Dendrophryniscus*) *stelzneri*, found in Paraguay and Argentina, is described by D. Cochran [*Living Amphibians of the World* (Doubleday, Garden City, N.Y., 1969)] as having poisonous skin. J. S. Budgett [*Quart. J. Microscop. Sci.* **42**, 305 (1899)] relates that this brightly colored frog (designated *Phryniscus nigricans* by him) was shunned by a grass snake (*Paludicola signifera*) that readily devoured an agile olive-green frog in the same cage. According to H. B. Cott [*Adaptive Coloration in Animals* (Oxford Univ. Press, New York, 1940)], Professor Graham Kerr found that his pet bird, a crested screamer (*Cariama cristata*), while out for a walk, enjoyed eating many amphibians but always found *Atelopus stelzneri* to be highly unpalatable. We know of no experiments on the nature of the poison in this atelopid.
7. J. Schindelman, M.S. thesis, Stanford University (1969); ———, F. A. Fuhrman, H. S. Mosher, *Toxicon*, in press.
8. B. K. Ranney, G. J. Fuhrman, F. A. Fuhrman, in preparation.
9. D. A. Rytand, *Ann. Int. Med.* **65**, 125 (1966).
10. F. A. Fuhrman and G. J. Fuhrman, in preparation.
11. H. Michl and E. Kaiser, *Toxicon* **1**, 175 (1963).
12. G. Habermehl, *Z. Naturforsch.* **20**, 1129 (1965).
13. V. Erspamer, T. Vital, M. Roseghini, J. M. Ceig, *Arch. Biochem. Biophys.* **105**, 620 (1964).
14. H. S. Mosher, F. A. Fuhrman, H. D. Buchwald, H. G. Fischer, *Science* **144**, 1100 (1964).
15. E. J. Schantz, J. M. Lynch, G. Vayvada, K. Matsumoto, H. Rapoport, *Biochemistry* **5**, 1191 (1966).
16. Supported by grants HD-00218 and GM-16031 from NIH.

26 May 1969; revised 4 August 1969 ■

Visual Stimuli and Evoked Responses in the Rat

Abstract. *Rats were presented with a series of visual stimuli, each described by shape, size, and position. Cortical recordings were made in medial and lateral positions of rat visual cortex. Average responses were calculated and correlated. This analysis revealed that information regarding size was contained in electrical activity from medial areas, and informtion regarding shape in electrical activity from lateral areas.*

Changes in visual stimuli to the rat usually result in perturbations of the electrical activity of certain areas of the cortex. The perturbation appears to end a short time after the change, and the section of the electroencephalograph record beginning with the stimulus and ending with the disappearance of the perturbation will be known as an individual evoked response (IER). If a particular change in a visual stimulus is repeated, and the resulting IER's are averaged, one may calculate an averaged evoked response (AER).

The changes in visual stimuli to the subject may be described by a number of observables. In these experiments black and white visual stimuli were used, so they can be completely defined by edge positions, lengths, and orientations. We may specify brightness and shape, with position and orientation, without significant loss of information.

Each visual stimulus is therefore a point in "stimulus space" whose location is specified by the values of the observables. Similarly, each IER is a point in function space. Since the IER's are measured with equipment of limited frequency response, the sampling theorem predicts that the functions can be represented by a finite number of samples of the finite record. The subject serves as a transform from stimulus space to "response space" within the limits of their definition. As our chosen observables will probably not completely define the stimulus, apparently equivalent stimuli need not map to the same point in response space, and never do. Rather, the points form a cluster whose "center" is approximated by the AER.

Several investigators have considered the information loss through this transformation. The AER or IER resulting from a given visual stimulus may be said to contain information regarding a particular observable if its position in response space changes when the value of that observable in stimulus space is changed. This may be difficult to determine because of uncontrolled variables producing unpredictable changes with repeated trials.

The AER to visual stimuli in monkeys (*1*) and in man (*2*) apparently contain more information about the shape parameter of the stimulus than about size or brightness parameters. The present study considers the relation between AER's and cortical recording sites in the rat.

Long-Evans hooded rats (male, 350 to 450 g) were used. Prior to the presentation of the stimuli they were curarized and respirated by the method of Miller and DiCara (*3*) and then placed in a stereotaxic instrument; Xylocaine was applied to the ears to minimize pain. While curare may have distorted the visual evoked responses, it seems unlikely that it could have been responsible for the effects reported. The rats were allowed to dark-adapt for 15 minutes before the presentations. While visual stimuli were being shown, the eye was observed with cross hairs and a dissecting microscope. No eye or pupil movements could be seen in the curarized rat, nor could any obvious deterioration of the eye be detected during the experiments.

The visual stimuli consisted of white shapes projected onto a dark background by a modified Sawyer projector. The projection bulb was replaced by the bulb of a Grass photostimulator, which allowed accurate control of the triggering of stimuli. The use of opaque slides showed that there were no appreciable electrical or acoustical arti-