mountains where the Blue Ridge escarpment runs nearly east to west and the ridge is cut by a number of narrow river valleys which form natural channels for warm, moist, southerly winds. The resultant high rainfall is reflected in luxuriant vegetation (*6*).

Gametophytes of the genus *Hymenophyllum* were found in all the river valleys examined, in some places 80 km (50 miles) away from the single site where the sporophyte is known in South Carolina. The gametophytic colonies of *Hymenophyllum* were often large, sometimes covering as much as a square meter of rock substrate. In a number of the colonies the gametophytes bore both archegonia and antheridia; nevertheless, sporophytes were encountered nowhere except in the original sporophyte locality. The gametophytes probably represent the same species as the sporophyte *Hymenophyllum tunbridgense* (L.) J. Sm. which grows in South Carolina.

The two vegetatively dispersed gametophytes which have been reported to grow in eastern United States in the absence of their sporophytic counterparts were also widely distributed in the Highlands area. One of these is the thallus known for over 30 years as the "Appalachian gametophyte" and more recently identified as probably the sexual stage of *Vittaria lineata* (L.) J. E. Sm. (*1*). The gametophyte grows as far north as Virginia and Ohio, but the sporophyte is known in the United States only in Florida and in one isolated station in Georgia. Identification of the other gametophyte, *Trichomanes*, reported from Illinois and Virginia (*2*, *7*), is more problematical, and, at least at Highlands, there may be several taxa involved. The identification is complicated because at least five species of *Trichomanes* in eastern United States probably have nearly identical gametophytes. Three of these, *T. punctatum* Poiret, *T. kraussii* Hook. and Grev., and *T. lineolatum* (v.d.B.) Hook., grow on rocks and mossy roots in southern Florida. The more temperate outliers of this primarily tropical genus are *T. petersii* A. Gray, which occurs from Louisiana and Florida north to the southern Appalachians (*5*), and *T. boschianum* Sturm, which is scattered in east-central United States (*8*). The *Trichomanes* in the Highlands area may be a combination of these species, but we found no means of distinguishing them with certainty.

The above-mentioned types of gametophytes may be expected to occur in a typical rain forest in tropical America on mossy tree trunks and rocks. In such areas, a fourth type is normally present, the elongate-cordate thallus type of the Grammitidaceae with its very distinctive gemmae. Although the possibility that this type of gametophyte might also exist at Highlands was entertained only as a remote one, it was indeed discovered there growing in large numbers behind a waterfall near the biological station. The identification was confirmed by the presence of about a dozen obviously juvenile or dwarfed sporophytes belonging to the species *Grammitis nimbata* (Jenm.) Proctor (*9*); the nearest locality for this species is Cuba, about 1280 km (800 miles) to the south. This is a remarkable new record for temperate North America. Possibly, this entire colony in North Carolina may have originated from the chance landing of a single spore from the south. The gametophyte has flourished locally by vegetative reproduction, but the sporophytes, judging from those growing at this single location, are unable to mature to their distinctive adult form. The tiny sporophytes, all less than 3 cm tall, entirely lacked reproductive structures.

On the basis of the foregoing observations, similar searches for clonal gemmiferous gametophytes might be made elsewhere, such as the temperate forest areas of Europe and eastern Asia. In the United States these gametophytes are usually found on damp, acidic rocks where the light intensity is too low for the growth of most bryophytes. Any gametophytes with gemma propagation may have the potential to exist continuously at long distances from their ancestral sporophyte populations.

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Ciguatoxin: Isolation and Chemical Nature

Abstract. *Ciguatoxin, the agent responsible for ciguatera, a disease produced in humans from ingestion of certain fishes, has been isolated from specimens of the moray eel,* Gymnothorax javanicus. *The toxin is apparently a lipid containing quaternary nitrogen, hydroxyl, and carbonyl functions.*

Ciguatera is a disease characterized by gastrointestinal and neurological symptoms and caused by ingestion of any one of a variety of tropical marine fishes. Although ciguatera was first reported as occurring in the Caribbean, most recent investigations on this disease have been made from occurrences in the Pacific (*1*). Unlike tetrodotoxin, which is probably produced by a single family of fishes (puffers), or unlike saxitoxin, which appears to originate in the phytoplankton and is found in plankton feeders such as clams and mussels, ciguatoxin, the agent which causes ciguatera, is found in a wide variety of fishes throughout the Pacific and Caribbean. Randall (*2*) and others have postulated that the toxin originates in a benthic alga and is transmitted through the food chain. Furthermore, the toxin-bearing fish may disappear from a given area and appear in an area previously considered free of poisonous fish. During the course of our work, toxicity of the red snapper, *Lutjanus bohar* (Forskål), has markedly decreased at Palmyra in the Line Islands and increased in the Marquesas Islands (*3*). In the Gilbert Islands the same species of fish is highly toxic on one reef and nontoxic on another reef a few hundred yards distant (*4*). Conditions in areas, such as the Line Islands, with no history of toxicity for over 100 years prior to the late 1930's, may suddenly change so that

most of the reef fish become highly toxic within a few months (*5*). At Johnston Island, about 1100 km southwest of Hawaii where 45 of 60 species of reef fish tested were reported toxic in 1954 (*6*), only the moray eel, *Gymnothorax javanicus* (Bleeker), and the shark, *Carcharhinus menisorrah* (Muller and Henle) (*7*), alone are now toxic.

We have described a procedure for screening fish from a toxic area; this procedure consists of feeding to mongooses (*1*), or a bioassay for the toxic extracts (*8*), or both. We have also reported (*9*) that ciguatoxin exhibits anticholinesterase activity in animals. We now describe the isolation and chemical nature of the toxin from the moray eel (*10*).

The toxin is extracted from 2 kg of raw flesh as follows. The flesh is treated with acetone at 20°C, and the insoluble (inactive) part is discarded. The acetone extract is concentrated and distributed between ether and water. The ether-soluble portion, which is now about 6 percent by weight, is concentrated and taken up in acetone at −20°C. The inactive precipitate contains the bulk of the phospholipids. The toxic acetone solution is concentrated, and the concentrate is taken up in methanol. The methanol-insoluble oil (inactive) is removed, and the methanolic solution (upon concentration the yield is about 0.030 percent) is extracted with *n*-hexane. The inactive hexane phase is discarded, and the methanol solution is concentrated to furnish about 0.015 percent of a toxic oil, corresponding to 300 mg (150 ppm), with a minimum lethal dose (MLD) of 30 mg/kg by intraperitoneal injection. From this toxic oil we removed inactive lipid material by column chromatography on silicic acid at 5°C, and elution with chloroform. Upon addition of 1 to 6 percent of methanol, active fractions were eluted with a recovery of 85 percent. We achieved further purification by preparative thin-layer chromatography (TLC) at 5°C on silica gel G in a mixture of chloroform, methanol, and *6N* ammonium hydroxide (90 : 9.5 : 0.5 by volume). After this chromatography process was repeated three times, the amount of toxic material was 1.5 mg of a new methanol-soluble oil. The toxicity (MLD) of the most active fraction was 0.5 mg/kg, as determined by intraperitoneal injection into mice (closed Carworth Farms Webster strain, 19 to 21 g). The observed symptoms in mice (diarrhea, excessive salivation, convulsive spasms, followed by respiratory failure) were identical to those produced by intermediate fractions. Titrimetric studies in our laboratory have further shown that crude and purified fractions exhibit parallel anticholinesterase activity, which differ only in degree of inhibition.

Ciguatoxin after preparative TLC is a transparent, light yellow, viscous oil which we were not able to crystallize. It is relatively unstable and loses toxicity in contact with air, light, and chromatographic adsorbents (alumina, Florisil, or silicic acid). To some extent, activity is lost even when the sample is stored in chloroform solution in the dark at −20°C. Although ciguatoxin behaves homogeneously on successive TLC plates in different solvent systems, the fact that we have not obtained a stable crystalline derivative keeps open the question whether ciguatoxin is a single substance with a tendency to decompose or whether it consists of several closely related compounds.

Combustion data obtained by ultramicro methods (*11*) indicate an empirical formula of $C_{35}H_{65}NO_8$ if there is only one nitrogen atom. Our earlier assumption that the molecule contains phosphorus was disproved by subsequent quantitative phosphorus analyses in two independent laboratories (*12*). Spectroscopic data confirm and expand our knowledge of the chemical nature. The highly saturated character of the compound is indicated by end absorption in the ultraviolet, by infrared bands at 2924, 2849, 1460, and 1379 cm^{-1}, and by a large NMR (nuclear magnetic resonance) peak centered at 1.25 ppm (*13*). The quaternary nature of the nitrogen atom is suggested by a positive Dragendorff test and a negative ninhydrin reaction. The functionality of the oxygen atoms is threefold. Infrared absorption at 3390 cm^{-1} indicates presence of hydroxyl; infrared absorption at 1742 cm^{-1} implies the presence of ester or cyclopentanone groups or both. The latter function is confirmed by a weak ultraviolet band at 270 $m\mu$ and by a positive 2,4-dinitrophenylhydrazine test. The ester function is amply demonstrated by spectrophotometric determination of the ferric hydroxamate complex (*14*) and by hydrolytic experiments. Hydrolysis of ciguatoxin in 2*N* hydrochloric acid in methanol at 70°C for 7 hours led to a fraction soluble in petroleum ether; infrared spectrum of this fraction points to the presence of nonhydroxylic long-chain fatty acid esters and to a chloroform-soluble fraction with a component giving a positive Dragendorff reaction (tertiary or quaternary nitrogen) and a positive reaction with 2,4-dinitrophenylhydrazine. Tests for a choline moiety in the hydrolyzate were negative. We established the presence of glycerol in the aqueous fraction by comparison (TLC and NMR) with an authentic sample.

Ciguatoxin may therefore be considered to be a lipid containing a quaternary nitrogen atom, one or more hydroxyl groups, and a cyclopentanone moiety. The fact that it is not a phosphatidic ester is of interest in connection with the established anticholinesterase activity (*9*).

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