Chapter 39

Chemistry and Pharmacology of Frog Venoms

JOHN W. DALY AND BERNHARD WITKOP

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES,
NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

I. INTRODUCTION

The pharmacologically active substances contained in the cutaneous secretions of various amphibians (Michl and Kaiser, 1963) appear to have evolved in this class of animals as agents which are employed more or less passively in defense against predators. These substances belong to many chemical classes and possess a wide range of pharmacological activities. Bufodienolides from toads of the genus *Bufo* (Chapters 37, 40), samandarine alkaloids from the European fire salamander (*Salamandra maculosa*) (Chapter 42), tetrodotoxin from newts of the genus *Taricha* (Mosher *et al.*, 1964), and batrachotoxin from the Colombian poison arrow frog (*Phyllobates aurotaenia*) (Märki and Witkop, 1963) are all extremely toxic compounds. Serotonin from various amphibians (Erspamer, 1954), bradykinin from the European grass frog (*Rana temporaria*) (Anastasi *et al.*, 1965), and the

various histamines and leptodactyline from the giant leptodactylid frog of Central and South America (*Leptodactylus pentadactylus*) (Erspamer *et al.*, 1964d) are better considered local irritants than poisons.

These various substances may serve merely as passive protective venoms or they may have other, more physiological functions in amphibians. It is noteworthy that the occurrence of such active substances in amphibians is often accompanied by gaudy warning coloration. In the diurnal frogs of the family *Dendrobatidae*, only the genera *Phyllobates* and *Dendrobates* are brightly colored, and only these frogs contain toxic principles; the closely allied dull-colored frogs of the genus *Colostethus* contain relatively inactive substances (see below).

Attempts to correlate the bright coloration and the level of toxicity in skin extracts were, however, unsuccessful with some 7 populations of a small Panamanian frog, *Dendrobates pumilio*. The range in general coloration in these frog populations was from dark blue, through various greens, reds, and reddish orange to bright red (Daly and Myers, 1967).

Correlation of serotonin levels in frog skin with habits of frogs of the genus *Rana* was more successful (Welsh and Zipf, 1966). High levels of serotonin were found only in the semiterrestrial species which might be more exposed to predators than their aquatic relatives.

Toads of the genus *Bufo*, although of dull coloration, are mainly terrestrial and have developed well-defined parotid glands capable of discharging their contents with some force. In these toads, the pharmacologically active compounds of the gland include cardioactive bufodienolides (Chapter 40), a variety of 5-hydroxyindolic amines (Cei *et al.*, 1968), and epinephrine (Abel and Macht, 1912); these compounds probably provide good protection against many predators. It has been observed that dogs promptly drop toads because of the effects of the gland secretions. The profuse skin secretions of the brightly colored European toads of the genus *Bombina* are reported to afford protection against natural predators such as snakes (Kiss and Michl, 1962). Preliminary experiments with Dendrobatid frogs indicate that frog-eating snakes, e.g., *Rhadinaea* promptly reject *Dendrobates pumilio* and *D. auratus* and then attempt to cleanse their buccal tissue (C. W. Myers, personal communication).

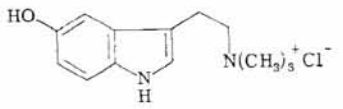
II. GENERAL CLASSES OF PHARMACOLOGICALLY ACTIVE SUBSTANCES FROM AMPHIBIANS

A. Biogenic Amines

This class of compounds occurs in many amphibians and has been extensively studied by Erspamer, Cei and co-workers. These amines occur

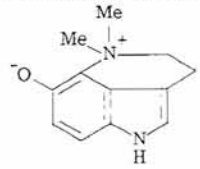
in various anurans at levels of greater than 1 mg/gm skin and may serve as a method for chemical taxonomy of certain amphibians (Cei *et al.*, 1967, 1968).

One of the most commonly occurring amines is serotonin and its *N*-methyl derivatives, *N*-methylserotonin, bufotenine, and bufotenidine (I). This type of compound is common in many anurans from the genera *Hyla*



(I)

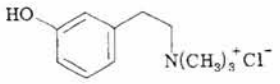
(Erspamer *et al.*, 1966; De Caro *et al.*, 1968), *Leptodactylus* (Cei *et al.*, 1967), *Rana* (Welsh and Zipf, 1966), and *Bufo* (Cei *et al.*, 1968). Certain anurans of these genera, such as *Hyla pearsoniana*, and *Leptodactylus pentadactylus*, have levels of 5-hydroxyindolethylamines ranging from 1–10 mg/gm skin, while, in other genera of these compounds may be detected in low concentration or not at all. 5-Hydroxyindolic amines also have been reported (Cei *et al.*, 1967, 1968; Erspamer 1954; Erspamer *et al.*, 1966b; Cei and Erspamer, 1966; Welsh and Zipf, 1966) in *Rhinoderma, Odontophrynus, Discoglossus, Bombina, Xenopus, Pleurodema, Eleutherodactylus, Melanophrynicus, Cyclorana,* and *Lechriodus*. In most of these anurans, serotonin or bufotenine is the major indolic material. In toads of the genus *Bufo*, a wider spectrum of amines occur (Cei *et al.*, 1968), these include dehydrobufotenine (II), its sulfate ester, bufothionine, and the sulfate ester, bufoviridine, of bufotenine. A hallucinogenic substance (Gessner *et al.*, 1968), 5-methoxy-*N*,*N*-dimethyltryptamine, is present in extremely high levels in *Bufo alvarius* (Erspamer *et al.*, 1967), but not in other toads of the same genus.



(II)

Serotonin has been reported (Erspamer, 1954) to be present in the European fire salamander (*Salamandra maculosa*) but not in certain other caudates [*Notophthalmus viridescens* (Welsh and Zipf, 1966), *Triturus pyrrhogaster* (Erspamer *et al.*, 1964c), *Ambystoma tigrinum, Triturus cristatus, Pleurodeles waltlii, Euproctes rusconi* (Erspamer, 1954), *Necturus maculosus* (Welsh, 1964)]. Tryptamine was reported (Erspamer 1954) in both *Salamandra maculosa* and *Triturus cristatus*.

The phenolic quarternary amine, leptodactyline (III) is also widespread

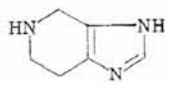


(III)

among anurans. It appears in many species (Cei *et al.*, 1967) of the genera *Pleurodema* and *Leptodactylus* and is also reported in *Telmatobus, Calyptocephalella, Elosia, Thoropa, Eupsophus, Physalaemus, Ceratophrys, Lepidobatrachus,* and *Odontophrynus*. It was first discovered in *Leptodactylus ocellatus* (Erspamer, 1959) in levels as high as 9 mg/gm skin (Erspamer *et al.*, 1964c). In addition to leptodactyline, the skin of *Leptodactylus pentadactylus pentadactylus* contains the *p*-hydroxy isomer, candicine, and small amounts of tyramine (Erspamer *et al.*, 1963, 1964c).

Extracts from the glands of *Bufo marinus* and certain other toads of this genus contain catecholamines consisting, principally, of epinephrine with lesser amounts of norepinephrine, dopamine, and epinine (*N*-methyldopamine) (Chen and Chen, 1934, 1951, Oestlund, 1954, Henderson *et al.*, 1960, Märki *et al.*, 1962). Epinephrine was not detected in all toads of this genus (Chen and Chen, 1934; Henderson *et al.*, 1962).

Histamine is found in certain anurans from the genera *Leptodactylus* (Cei *et al.*, 1967) and *Hyla* (Erspamer *et al.*, 1966b). Skin extracts from *Leptodactylus pentadactylus labyrinthicus* contain, in addition to histamine, *N*-methylhistamine, *N*-acetylhistamine, and *N,N*-dimethylhistamine, 2 bicyclic amines, spinaceamine (IV), and 6-methylspinaceamine (Erspamer *et al.*, 1964c,d). A summary of the biogenic amines and other active substances



(IV)

in various anurans is presented in Tables I–IV. Most of the amines, because of their effects on buccal and mucous tissue, could serve as passive defensive agents against predators.

B. Peptides

A variety of peptides, many with extremely high toxic activity, have been isolated from extracts of various frogs. These include bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), physalaemin (Pyroglutamyl-Ala-Asp-Pro-Asp(NH_2)-Lys-Phe-Tyr-Gly-Leu-Met-NH_2), and caerulein (Pyroglutamyl-Glu-Asp-Tyr-(SO_3H)-Thr-Gly-Tryp-Met-Asp-Phe-NH_2).

Physalaemin was first discovered in *Physalaemus fuscumaculatus* (Erspamer *et al.*, 1964a) and has also been identified in *P. centralis*. It

produces an extreme vasodilatation and prolonged hypotension. It is characterized by its immediate stimulant action on extravascular smooth musculature which contrasts with the slower action of bradykinin-like peptides. Physalaemin-like polypeptides have also been detected in *Physalaemus bresslaui* and *P. cuvieri* and in *Phyllomedusa rohdei, P. hypochondrialis,* and 4 other species of *Phyllomedusa,* in *Telmatobius jelskii* (Bertaccini *et al.,* 1965; Erspamer and Anastasi, 1965), and in *Uperoleia rugosa* and *U. marmorata* (Erspamer *et al.,* 1966a). In the latter species the name "Uperolein" has been suggested for the hypotensive polypeptide.

Bradykinin was first discovered in amphibians in extracts from the skin of *Rana temporaria* (Anastasi *et al.,* 1965a). It is also present in other frogs of this genus (*R. nigromaculata*) (Nakajima, 1968). Bradykinin-like peptides have been noted in *Rana esculenta* (Erspamer and Anastasi, 1965) and other *Ranae* (Erspamer *et al.,* 1964c), in *Phyllomedusa rohdei* (bradykinyl-Ile-Tyr-(SO_3H) = phyllokinin) (Anastasi *et al.,* 1965b), in *Rana nigromaculata* (Val-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg = 1-Val-6-Thr bradykinin) (Nakajima, 1968), and in *Ascaphus truei* (Erspamer and Anastasi, 1965). *Acaphus truei* contains very large amounts of a bradykinin-like peptide which may be identical with phyllokinin. The activity of bradykinin as a hypotensive agent, a smooth muscle stimulant, and an irritant is well known.

Caerulein was first isolated from *Hyla caerulea* (Anastasi *et al.,* 1968). Caerulein or caerulein-like peptides occur in many species of *Hyla* (De Caro *et al.,* 1968), in certain species of *Leptodactylus* (*L. rubido, curtus, pentadactylus, laticeps*) (Cei *et al.,* 1967), and in certain *Phyllomedusae* (Erspamer and Anastasi, 1965). Caerulein is a hypotensive agent and a smooth muscle stimulant and, in addition, it stimulates gastric and pancreatic secretions.

Certain other peptides with either novel or unknown pharmacological activity have been isolated from frog skin extracts. These include: carnosine from various species of *Eleutherodactylus* (*E. portoricensis, E. karlschmidti, richmondi, wightmanae, locustus, eneidae, gryllus,* and *longirostris*), *Leptodactylus albilagrius* (Daly and Heatwole, 1966), and *Dendrobates pumilio*; tryptokinins from *Phyllomedusa rohdei* and *P. hypochondrialis* (Erspamer and Anastasi, 1965); and a hexapeptide [Ala-Glu-His-Phe-Ala-Asp $(NH_2)_2$] from *Bombina variegata* (Kiss and Michl, 1962).

Because of their stimulation of smooth muscle, many of these peptides could serve the frog, in defense, as chemical irritants, but the possibility exists that they may also have a physiological function. The occurrence of active peptides in various anurans is presented in Tables I–III.

C. Proteins

Many anurans contain extremely toxic hemolytic proteins in their skin secretions. Earlier investigations also had demonstrated such proteins in extracts of certain salamanders (*Salamandra maculosa, Triturus vulgaris, T.*

TABLE I

THE OCCURRENCE OF PHARMACOLOGICALLY ACTIVE SUBSTANCES IN EXTRACTS OF SKIN FROM VARIOUS ANURANS[a]

	Serotonin	*N*-Methyl-serotonin	Bufotenine	Bufotenidine	Bufoviridine	Dehydrobufotenine	Bufothionine	Leptodactyline	Histamine	Active peptides	Epinephrine	Hemolytic proteins	Steroidal alkaloids
Ascaphidae													
Ascaphus truei	—	—	—	—	—	—	—	—	—	B	—	—	—
Pseudidae													
Pseudis paradoxus	○	—	—	—	—	—	—	—	—	—	—	—	—
Discoglossidae													
Discoglossus pictus	++	○	○	○	○	○	○	—	—	—	—	—	—
Bombina variegata	++	○	○	○	○	○	○	—	—	×	—	×	—
B. bombina	+	○	○	○	○	○	○	—	—	—	—	—	—
Pipidae													
Xenopus laevis	+	○	○	++	○	○	○	—	—	—	—	—	—
Pelobatidae													
Pelobates fuscus	○	○	○	○	○	○	○	—	—	—	—	×	—
Bufonidae[b]													
Bufo regularis	+++[c]	○	○	○	○	○	○	○	○	○	×	—	—
B. mauretanicus	++	○	○	○	○	○	○	○	○	○	×	—	—
B. kisoloensis	++	○	○	○	○	○	○	○	○	○	—	—	—
B. berghei	++	○	○	○	○	○	○	○	○	○	—	—	—
B. funereus	++	○	○	○	○	○	○	○	○	○	—	—	—
B. melanostictus	++	○	+	+++	○	+	○	○	○	○	○	—	—

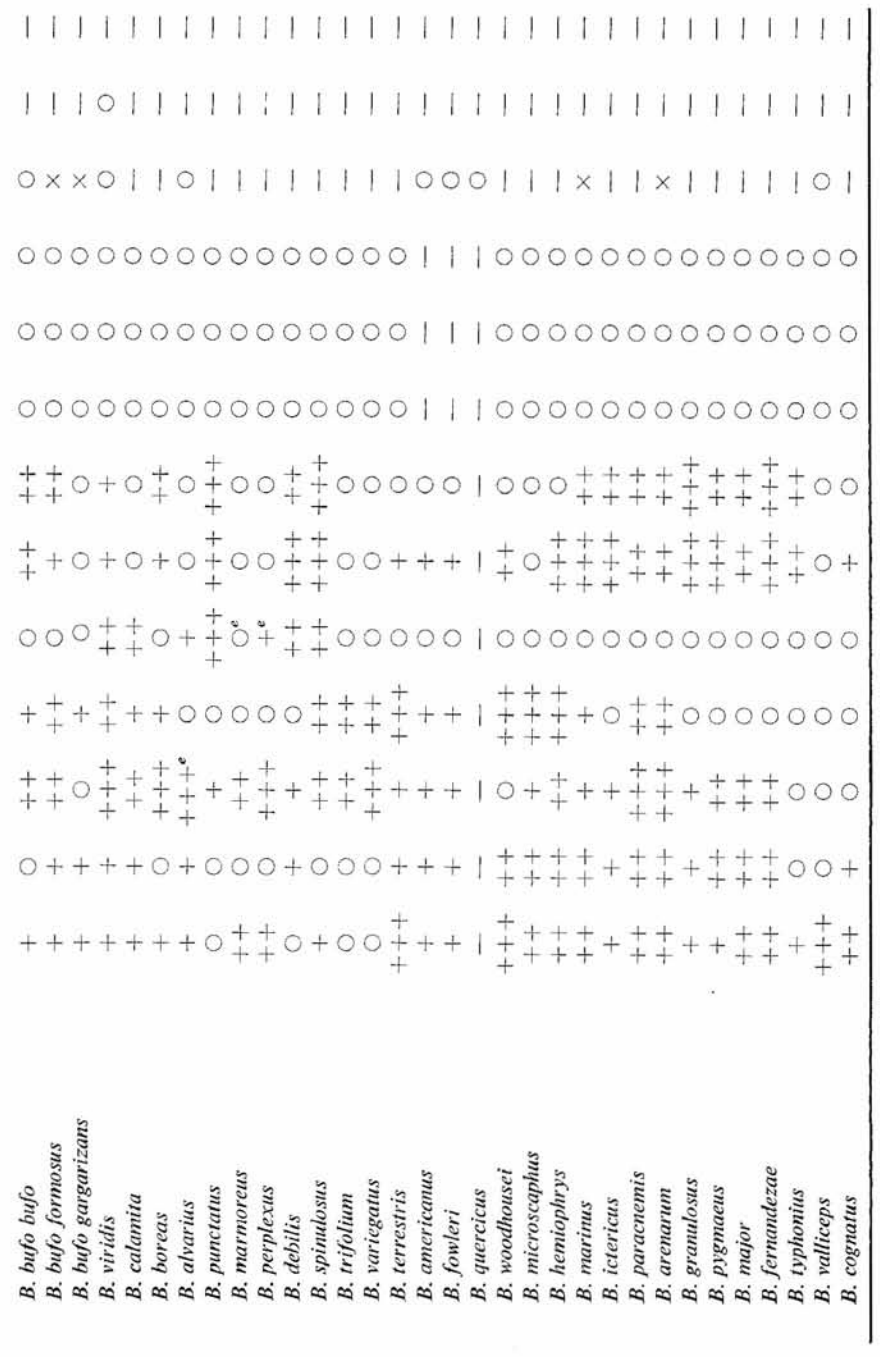

B. bufo bufo
B. bufo formosus
B. bufo gargarizans
B. viridis
B. calamita
B. boreas
B. alvarius
B. punctatus
B. marmoreus
B. perplexus
B. debilis
B. spinulosus
B. trifolium
B. variegatus
B. terrestris
B. americanus
B. fowleri
B. quercicus
B. woodhousei
B. microscaphus
B. hemiophrys
B. marinus
B. ictericus
B. paracnemis
B. arenarum
B. granulosus
B. pygmaeus
B. major
B. fernandezae
B. typhonius
B. valliceps
B. cognatus

TABLE I—(*continued*)

	Serotonin	*N*-Methyl-serotonin	Bufotenine	Bufotenidine	Bufoviridine	Dehydrobufotenine	Bufothionine	Leptodactyline	Histamine	Active peptides	Epinephrine	Hemolytic proteins	Steroidal alkaloids
B. speciosus	+++	++	○	○	○	+	○	○	○	○	—	—	—
B. canaliferus	+++	++	○	+	○	○	++	○	○	○	—	—	—
B. coccifer	++	+	○	+++	○	○	○	○	○	○	—	—	—
B. luetkeni	++	+	+	○	○	○	○	○	○	○	—	—	—
B. haematiticus	+++	○	○	○	○	+++	○	○	○	○	—	—	—
B. crucifer	○	○	+	○	○	○	○	—	—	—	×	—	—
B. blombergi	—	—	—	—	—	—	—	—	—	—	×	—	—
B. peltocephalus	—	—	—	—	—	—	—	—	—	—	×	—	—
B. asper	—	—	—	—	—	—	—	—	—	—	○	—	—
Atelopodidae													
Melanophryniscus moreirae	+	+	+++	○	○	○	○	○[f]	○	○	—	—	—
M. stelzneri	○	○	+	○	○	○	○	○	○	○	—	—	—

[a] The data reported are taken from references cited in the text: — = No data available; ○ = not present or less than 1 μg/gm; + = 1–100 μg/gm; ++ = 100–1000 μg/gm; +++ = 1–10 mg/gm; × = present in extracts; P = physaelamin-like peptides; B = bradykinin-like peptides; C = caerulin-like peptides.

[b] Toads of the genus *Bufo* contain cardiotoxic bufodienolides (Chapters 38, 40).

[c] Contains the *O*-sulfate of serotonin.

[d] Contains *N*-methyl-5-methoxytryptamine, the N_1-sulfate of bufotenine, the *N*-sulfate of 5-methoxy-*N*,*N*-dimethyltryptamine, and enormous amounts of 5-methoxy-*N*,*N*-dimethyltryptamine (50–160 mg/gm skin).

[e] Contains the N_1-sulfate of bufotenine in large amounts (>500 μg/gm skin).

[f] Contains a phenolic amine.

TABLE II

THE OCCURRENCE OF PHARMACOLOGICALLY ACTIVE SUBSTANCES IN EXTRACTS OF SKIN FROM VARIOUS ANURANS[a]

	Serotonin	*N*-Methyl-serotonin	Bufotenine	Bufotenidine	Bufoviridine	Dehydrobufotenine	Bufothionine	Leptodactyline	Histamine	Active peptides	Epinephrine	Hemolytic proteins	Steroidal alkaloids
Leptodactylidae													
Batrachophrynus macrostonum	○	○	○	○	○	○	○	○	○	○	—	—	—
B. patagonicus	○	○	○	○	○	○	○	○	○	○	—	—	—
Telmatobius montanus	○	○	○	○	○	○	○	○	○	○	—	—	—
T. hauthali	○	○	○	○	○	○	○	○	○	○	—	—	—
T. halli	○	○	○	○	○	○	○	○	○	○	—	—	—
T. jelskii	○	○	○	○	○	○	○	+ ○	○	P	—	—	—
Calyptocephalella gayi	○	○	○	○	○	○	○	○	○	○	—	—	—
Elosia aspera	○	○	○	○	○	○	○	○	○	○	—	—	—
E. lateristrigata	○	○	○	○	○	○	○	○	○	○	—	—	—
Cyclorhamphus fuliginosus	○	○	○	○	○	○	○	○	○	○	—	—	—
Thoropa miliaris	+	○	○	○	○	○	○	+	○	○	—	—	—
T. petropolitana	○	○	○	○	○	○	○	○	○	○	—	—	—
Eupsophus rosens	○	○	○	○	○	○	○	○	○	○	—	—	—
E. nodosus	○	○	○	○	○	○	○	○	○	○	—	—	—
Eleutherodactylus ranoides	○	○	○	○	○	○	○	○	○	○	—	—	—
E. bufoniformis	○	○	○	○	○	○	○	○	○	○	—	—	—

TABLE II—(*continued*)

	Serotonin	*N*-Methyl-serotonin	Bufotenine	Bufotenidine	Bufoviridine	Dehydrobufotenine	Bufothionine	Leptodactyline	Histamine	Active peptides	Epinephrine	Hemolytic proteins	Steroidal alkaloids
E. portoricensis	○	○	○	○	○	○	○	○	○	○[b]	—	—	—
E. martinicensis	+	—	—	—	—	—	—	—	—	—	—	—	—
Rhinoderma darwini	++	○	○	○	○	○	○	○	○	○	—	—	—
Physalaemus biligonigerus	○	○	○	○	○	○	○	+	○	**P**	—	—	—
P. fuscumaculatus	—	—	—	—	—	—	—	—	—	**P**	—	—	—
P. centralis	○	○	○	○	○	○	○	○	○	**P**	—	—	—
P. bresslaui	○	○	○	○	○	○	○	○	○	**P**	—	—	—
P. cuvieri	○	○	○	○	○	○	○	○	○	**P**	—	—	—
Eupemphix nattereri	○	○	○	○	○	○	○	○	○	○	—	—	—
Pleurodema cinerea	○	○	○	○	○	○	○	+	○	○	—	—	—
P. tucuma	○	○	○	○	○	○	○	+	○	○	—	—	—
P. quayapae	○	○	○	○	○	○	○	○	○	○	—	—	—
P. neubulosa	○	○	○	○	○	○	○	○	○	○	—	—	—
P. bibroni	○	○	○	○	○	○	○	+	○	○	—	—	—
P. bufonina	+	○	○	○	○	○	○	○	○	○	—	—	—
Leptodactylus bufonicus	○	○	○	○	○	○	○	+	○	○	—	—	—
L. prognathus	○	○	○	○	○	○	○	+	○	○	—	—	—
L. sibilatrix	○	○	○	○	○	○	○	+	○	○	—	—	—
L. gracilis	○	○	○	○	○	○	○	+	○	○	—	—	—
L. mystacinus	○	○	○	○	○	○	○	+	○	○	—	—	—
L. caligonosus	++	○	○	+	○	○	○	+++	○	○	—	—	—
L. melanonotus	+	+	○	++	○	○	○	++	○	○	—	—	—
L. rubido	+	○	○	+	○	○	○	++	○	**C**	—	—	—

L. curtus	+	○	○	+	○	○	○	+++	○	C	—	—	—
L. pentadactylus	++	○	○	++[c]	○	○	○	+[d]	++[e]	C	—	—	—
L. laticeps	++	○	○	○	○	○	○	+	++[f]	C	—	—	—
L. bolvianus	○	○	○	○	○	○	○	++	○	○	—	—	—
L. ocellatus	○	○	○	○	○	○	○	+++	○	○	—	—	—
L. chaquensis	○	○	○	○	○	○	○	++	○	○	—	—	—
Ceratophrys ornata	○	○	○	○	○	○	○	+	—	—	—	—	—
Lepidobatrachus asper	○	○	○	○	○	○	○	+	—	—	—	—	—
L. salinicola	○	○	○	○	○	○	○	+	—	—	—	—	—
L. llanensis	○	○	○	○	○	○	○	○	—	—	—	—	—
Odontophrynus americanus	++	○	○	○	○	○	○	+	—	—	—	—	—
O. occidentalis	+++	○	○	○	○	○	○	+	—	—	—	—	—
Adelotus brevis	○	○	○	○	○	○	○	○	○	○	—	—	—
Mixophyes fasciolatus	○	○	○	○	○	○	○	○	○	○	—	—	—
Limnodynastes fletcheri	○	○	○	○	○	○	○	○	○	○	—	—	—
L. ornatus	○	○	○	○	○	○	○	○	○	○	—	—	—
L. peroni	○	○	○	○	○	○	○	○	○	○	—	—	—
Cyclorana alboquttatus	+	++	○	○	○	○	○	○	○	○	—	—	—
Lechriodus fletcheri	+	○	○	○	○	○	○	○	○	○	—	—	—
Uperoleia rugosa	—	—	—	—	—	—	—	—	—	P	—	—	—
U. marmorata	—	—	—	—	—	—	—	—	—	P	—	—	—
Pseudophryne corroboree	—	—	—	—	—	—	—	—	—	—	—	—	+

[a] The data reported are taken from references cited in the text: — = No data available; ○ = not present or less than 1 μg/gm; + = 1–100 μg/gm; ++ = 100–1000 μg/gm; +++ = 1–10 mg/gm; × = present in extracts; P = physaelamin-like peptides; B = bradykinin-like peptides; C = caerulin-like peptides.

[b] Contains carnosine.

[c] *L. pentadactylus dengleri* (not in other subspecies).

[d] *L. pentadactylus pentadactylus* also contains candicine and tyramine.

[e] *L. pentadactylus labyrinthicus* also contains *N*-methylhistamine, *N,N*-dimethylhistamine, *N*-acetylhistamine, and (6-methyl)spinaceamine, and amounts of 5-hydroxytryptamine over 1 mg/kg.

[f] Contains spinaceamine.

TABLE III

THE OCCURRENCE OF PHARMACOLOGICALLY ACTIVE SUBSTANCES IN EXTRACTS OF SKIN FROM VARIOUS ANURANS[a]

	Serotonin	*N*-Methyl-serotonin	Bufotenine	Bufotenidine	Bufoviridine	Dehydrobufotenine	Bufothionine	Leptodactyline	Histamine	Active peptides	Epinephrine	Hemolytic proteins	Steroidal alkaloids
Dendrobatidae													
Phyllobates aurotaenia	○	○	○	○	○	○	○	○	○	—	—	—	×
P. vittatus	○	○	○	○	○	○	○	○	○	—	—	—	×
Dendrobates pumilio	○	○	○	○	○	○	○	○	○	—[b]	—	—	×
D. granuliferus	—	—	—	—	—	—	—	—	—	—	—	—	×
D. auratus	—	—	—	—	—	—	—	—	—	—	—	—	×
D. histrionicus	—	—	—	—	—	—	—	—	—	—	—	—	×
D. leucomelas	—	—	—	—	—	—	—	—	—	—	—	—	×
D. minutus	—	—	—	—	—	—	—	—	—	—	—	—	×
Hylidae													
Hyla arborea	+	○	○	○	○	○	○	○	○	×	—	×	—
H. aurea	+	○	○	○	○	○	○	—	—	—	—	—	—
H. caerulea	++	○	○	○	○	○	○	○	++	C	—	—	—
H. pearsoniana	+	○	+++	○	○	○	○	○	○	○	—	—	—
H. peroni	++	+	+	○	○	○	○	○	○	○	—	—	—

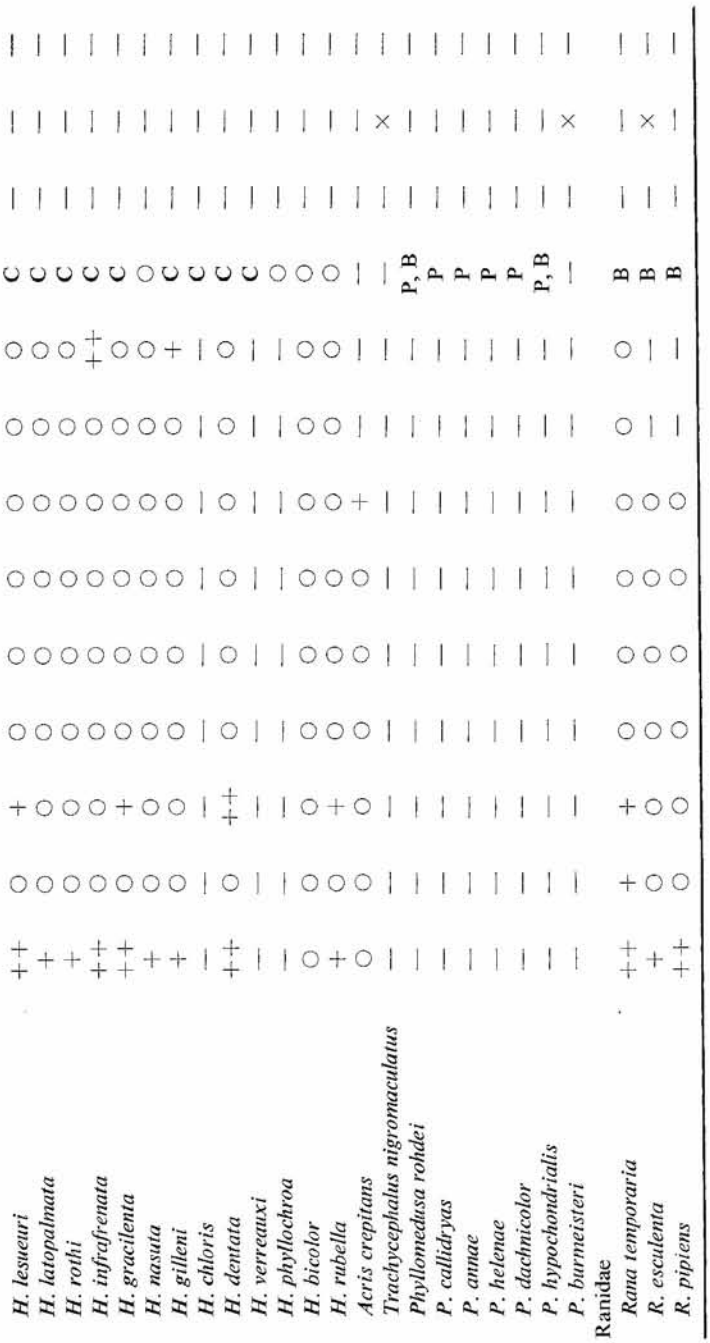

H. lesueuri
H. latopalmata
H. rothi
H. infrafrenata
H. gracilenta
H. nasuta
H. gilleni
H. chloris
H. dentata
H. verreauxi
H. phyllochroa
H. bicolor
H. rubella
Acris crepitans
Trachycephalus nigromaculatus
Phyllomedusa rohdei
P. callidryas
P. annae
P. helenae
P. dachnicolor
P. hypochondrialis
P. burmeisteri
Ranidae
Rana temporaria
R. esculenta
R. pipiens

TABLE III—(*continued*)

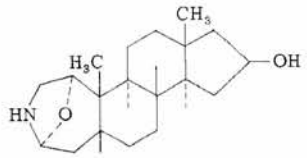
	Serotonin	*N*-Methyl-serotonin	Bufotenine	Bufotenidine	Bufoviridine	Dehydrobufotenine	Bufothionine	Leptodactyline	Histamine	Active peptides	Epinephrine	Hemolytic proteins	Steroidal alkaloids
R. palustris	+	○	○	○	○	○	○	—	—	—	—	—	—
R. labrosa	+	○	○	○	○	○	○	—	—	—	—	—	—
R. madagascariensis	+	○	○	○	○	○	○	—	—	—	—	—	—
R. fuscigola	○	○	○	○	○	○	○	—	—	—	—	—	—
R. latastei	+	—	—	—	—	—	—	—	—	○	—	—	—
R. dalmatina	+	—	—	—	—	—	—	—	—	○	—	○	—
R. sylvatica	++	—	—	—	—	—	—	—	—	—	—	—	—
R. aurora	++	—	—	—	—	—	—	—	—	—	—	—	—
R. sphenocephala	++	—	—	—	—	—	—	—	—	—	—	—	—
R. catesbeiana	○	○	○	○	○	○	○	○	○	B	—	—	—
R. grylio	○	—	—	—	—	—	—	—	—	—	—	—	—
R. clamitans	○	○	○	○	○	○	○	—	—	—	—	—	—
R. nigromaculata	+	○	○	○	○	○	○	○	○	B	—	—	—
R. japonica	+	○	○	○	○	○	○	○	○	B	—	—	—
R. rugosa	++	○	○	○	○	○	○	○	○	B	—	—	—
R. limnocharis	○	○	○	○	○	○	○	○	○	○	—	—	—
Arthroleptis adolphifriederici	○	○	○	○	○	○	○	—	—	—	—	—	—
Ptychadena mascariniensis	○	○	○	○	○	○	○	—	—	—	—	—	—
Rhacophoridae													
Rhacophorus madagascariensis	○	○	○	○	○	○	○	—	—	—	—	—	—
Chiromantis rufescens	○	○	○	○	○	○	○	—	—	—	—	—	—
Polypedates buergeri	○	○	○	○	○	○	○	—	—	○	—	—	—

[a] The data reported are taken from references cited in the text: — = No data available; ○ = not present or less than 1 μg/gm; + = 1–100 μg/gm; ++ = 100–1000 μg/gm; +++ = 1–10 mg/gm; × = present in extracts; P = physaelamin-like peptides; B = bradykinin-like peptides; C = caerulin-like peptides. [b] Contains carnosine.

cristatus, and *T. marmoratus*) (Michl and Kaiser, 1963). Such hemolytic proteins occur in various anurans, e.g., *Bombina variegata, Rana esculenta,* and *Hylo arborea* (Kiss and Michl, 1962). Partial purification of a hemolytic protein from *Pelotes fuscus* has been carried out (Lábler *et al.,* 1968). The occurrence of such hemolytic substances is presented in Tables I–III.

D. Steroidal Alkaloids

Extremely toxic steroidal alkaloids have now been found to occur not only in salamanders (Chapter 42) but also in anurans. The principal alkaloid, samandarine (V), from the European fire salamander *Salamandra maculosa* has now been demonstrated in an Australian anuran *Pseudophryne corroboree* (Habermehl, 1965). The steroidal alkaloids contained in a small dendrobatid frog (*Dendrobates pumilio*) were first regarded as similar in structure



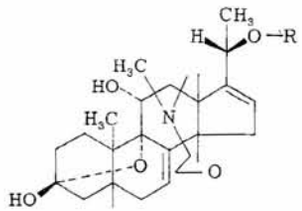
(V)

to samandarine (Daly and Myers, 1967), but subsequent studies have shown that they are a new type of alkaloid (see below).

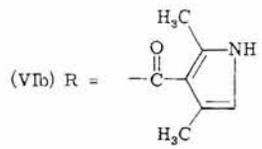
It has long been known that dendrobatid frogs contain very toxic skin secretions. In 1871, A. Posada Arango (1871) first described a poison used by the Noanama, Cuna, and Choco Indians of the Choco and Antioquia departments of Colombia for their blow darts. This poison was obtained from a milky secretion of a small frog which was given the name *Phyllobates chocoensis* by this author. Later studies by Santesson (1935) on this poison attributed its source to *Dendrobates tinctorius.* Santesson observed paralysis of the muscles and central nervous system, especially the respiratory centers, and finally systolic heart arrest. Mezey (1947), using venom from darts 15 years old, attributed it to a species of *Dendrobates,* observed dyspnea, bradycardia, paralysis of the hind limbs, and loss of equilibrium. He attributed death to respiratory failure and cardiotoxic effects resulting in hypotension. In our own studies (Märki and Witkop, 1963) on poison isolated directly from the skin of frogs, dyspnea and loss of equilibrium followed by violent intermittent convulsions and death were observed. Two varieties of frogs were used for these studies, and these were incorrectly identified first as *Phyllobates bicolor* (Märki and Witkop) and then as *P. latinasus* (Latham, 1966). The correct name is *Phyllobates aurotaenia* (Daly and Myers, 1967), a frog originally described by Boulenger (1913) as *Dendrobates aurotaenia.* It is possible that a variety of dendrobatid frogs from this region have been

used by Indians to poison their blow darts, but at the present time only the two varieties of *Phyllobates aurotaenia* seem to be employed. Parenthetically, Breder (1946) mentions the use of *Dendrobates auratus* as the former source of poison for blow darts by the Choco Indians of the Darien region of Panama.

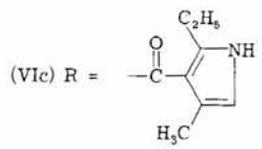
The chemical structure of these toxic principles has now been elucidated. The major venom of the Colombian poison arrow frog (*Phyllobates aurotaenia*) is batrachotoxin (VIb) (Märki and Witkop, 1963; Daly *et al.*, 1965; Tokuyama *et al.*, 1968, 1969). The structure of homobatrachotoxin, formerly called isobatrachotoxin, is shown below (VIc). Crystalline batrachotoxinin A (VIa), the much less active steroidal alcohol component of the esters (VIb, VIc) was used, as the *p*-bromobenzoate derivative, for a complete X-ray



(VIa) R = H



(VIb) R =



(VIc) R =

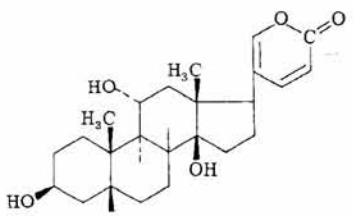
analysis. Such novel nitrogenous steroids have now been demonstrated not only in *Phyllobates aurotaenia* (Choco, Colombia) but also, in much smaller amounts, in *P. vittatus* (Southwestern Costa Rica) and are perhaps typical of this genus.

Dendrobates pumilio of the closely allied genus, *Dendrobates*, contains a different type of compound (Daly and Myers, 1967) whose simplest member is pumiliotoxin C, a bicyclic amine with the empirical formula $C_{13}H_{25}N$. Similar compounds have now been isolated from *D. auratus* and may be typical of this genus.

All these poisons are irritating to mucous and buccal tissues and in all likelihood provide a great deal of protection for these small brightly colored

terrestrial diurnal frogs. Toxic steroidal alkaloids have been reported for a limited number of genera (Tables I–III).

It is noteworthy that there are similarities (3,14β-oxygen functions, 18,19β-alkyl groups, and the β-6H) among the steroid moiety of batrachotoxin, the most active cardiotoxin known, and the cardioactive bufodienolides, e.g., (gamabufotalin VII), found in toads of the genus *Bufo* (Chapter 40).



(VII)

III. STEROIDAL ALKALOIDS FROM THE COLOMBIAN POISON ARROW FROG (*Phyllobates aurotaenia*)

The Colombian frog, *Phyllobates aurotaenia*, is employed by Choco Indians in Colombia to poison their blow darts. Two color varieties are employed: one a gleaming black frog with bright yellow dorsolateral stripes, the other, from higher areas in the mountains, a slightly larger black frog with either broad red orange dorsolateral stripes or, when the stripes are fused, a solid red-orange back. Each frog contains less than 50 μg of the toxic principles, a quantity sufficient to kill approximately 1000 mice when injected subcutaneously.

The active principles are isolated by extracting the skins with methanol, concentrating the extract, and partitioning with chloroform and water. The chloroform layer is then extracted with 0.1 *N* HCl. The aqueous acid is made basic with 1 *N* ammonium hydroxide, and the toxic alkaloids are then re-extracted into chloroform. To curtail losses of the rather labile active compounds, these operations should be carried out at 5°C. Thin-layer or column chromatography in silica gel yields the 4 major bases batrachotoxin, homobatrachotoxin (which supersedes the earlier incorrect terminology, isobatrachotoxin), pseudobatrachotoxin, and batrachotoxinin A (Tokuyama *et al.*, 1968, 1969). Results from high resolution mass spectrometry indicate that these toxic materials are closely related in structure and that they are steroidal alkaloids. Batrachotoxin, homobatrachotoxin, and pseudobatrachotoxin all appear to be isomers ($C_{24}N_{33}NO_4$), while batrachotoxinin A ($C_{24}H_{35}NO_5$) differed in its hydrogen and oxygen content. Batrachotoxin

and homobatrachotoxin exhibited characteristic ultraviolet spectra (λ_{max} 234, ε= 9200, 264, ε = 5100), an infrared absorption band at 1690 cm^{-1}, which indicated a carbonyl or vinyl ether grouping, and a positive Ehrlich test which indicated a (potential) pyrrole ring. Pure pseudobatrachotoxin was not isolated in sufficient quantity for n.m.r. or infrared analysis because it converts easily to batrachotoxinin A. Neither of these latter compounds exhibited ultraviolet absorption bands or a positive Ehrlich test.

When extracts from 5000 frogs (Latham, 1966) were processed, a total of 11 mg of pure batrachotoxin, 16 mg of homobatrachotoxin, 1 mg of pseudobatrachotoxin, and 42 mg of batrachotoxinin A were isolated. Batrachotoxin, homobatrachotoxin, pseudobatrachotoxin, and batrachotoxinin A appear to occur in this frog (*P. aurotaenia*) in a ratio of approximately 3:1:3:3. Most of the pseudobatrachotoxin is converted to batrachotoxinin A during isolation.

Because of the limited quantity of alkaloid available, attention was directed toward obtaining a suitable crystalline derivative for X-ray analysis. In 1967, batrachotoxinin A was converted with *p*-bromobenzoic anhydride, under Schotten-Baumann conditions, to a crystalline *O*-*p*-bromobenzoate which, on treatment with base, regenerated batrachotoxinin A. X-ray analysis of a single crystal of this derivative led to the complete structure of batrachotoxin A (VIa) (Tokuyama *et al.*, 1968).

Reexamination of n.m.r., ultraviolet, infrared, and mass spectral data of batrachotoxinin A, batrachotoxin, and homobatrachotoxin demonstrated that the ion at m/e = 399 ($C_{24}H_{33}NO_4$), formerly thought to be the parent ion of batrachotoxin and homobatrachotoxin, was, in fact, a fragment. These alkaloids appeared to contain the steroidal nucleus of batrachotoxinin A and an additional moiety which accounted for the characteristic ultraviolet spectra (infrared band at 1690 cm^{-1}), Ehrlich reaction, and the presence (n.m.r. spectra) of 2 additional methyl groups in batrachotoxin, or 1 methyl and 1 ethyl group in homobatrachotoxin. Examination of the mass spectra of batrachotoxin and homobatrachotoxin, from this point of view, led to the identification of a fragment due to this additional moiety at m/e 139 ($C_7H_9NO_2$) in batrachotoxin and m/e 153 ($C_8H_{11}NO_2$) in homobatrachotoxin. Since this additional moiety, $C_7H_9NO_2$, had to account for the ultraviolet chromophore, the infrared absorption band at 1690 cm^{-1}, the positive Ehrlich reaction, and the presence in the n.m.r. spectrum of peaks due to 2 additional aryl methyl groups, we concluded that batrachotoxin is a dimethylpyrrolecarboxylate of batrachotoxinin A and that homobatrachotoxin is the corresponding methylethylpyrrolecarboxylate. An attempt to demonstrate a true molecular ion for batrachotoxin was now successful and it was found, as expected, at m/e 538 ($C_{31}H_{42}N_2O_6$).

The ultraviolet absorption peaks of (homo)batrachotoxin were then

compared with spectra of dialkylated pyrrolecarboxylates and were found to be identical with that of ethyl 2,4-dimethylpyrrole-3-carboxylate. The n.m.r. studies confirmed that batrachotoxin and homobatrachotoxin were pyrrole-3- rather than 2-carboxylates. Investigation of changes in the chemical shift of the aryl methyl groups in different solvents provided n.m.r. evidence that homobatrachotoxin is a 2-ethyl-4-methylpyrrole-3-carboxylate rather than the other possible isomer 4-ethyl-2-methylpyrrole-3-carboxylate.

The point of attachment of the pyrrole carboxylate was also investigated. Hydrolysis with a strong base had been found to regenerate batrachotoxinin A. Evidence that the 20α-hydroxyl group was involved in the ester formation in batrachotoxin came from a comparison of the mass spectral fragmentation patterns and n.m.r. spectra of (homo)batrachotoxin and batrachotoxinin A. The n.m.r. resonance peak for the C-20 hydrogen was at $\delta = 5.8$–5.9 in (homo)batrachotoxin, an indication that the 20α-hydroxyl group was esterified.

The structures of batrachotoxin (VIb) and homobatrachotoxin (VIc) were confirmed by partial synthesis from batrachotoxinin A and the mixed anhydride prepared from 2,4-dimethylpyrrole-3-carboxylic acid and ethyl chloroformate. The synthetic batrachotoxin was identical in all respects (toxicity, n.m.r., infrared and mass spectra, color reactions, and chromotographic properties) with natural batrachotoxin (Tokuyama *et al.*, 1969).

Comparative toxicities of (homo)batrachotoxin, batrachotoxinin A, and their analogs are presented in Table IV along with samandarine and pumiliotoxin A and B.

TABLE IV

RELATIVE TOXICITY IN MICE OF NITROGENOUS BASES ISOLATED FROM ANURANS AND VARIOUS SYNTHETIC ANALOGS

	LD_{50} (μg/kg s.c.)
Batrachotoxin	2
Homobatrachotoxin	3
Batrachotoxinin A	1000
Batrachotoxinin A-20-(2,5-dimethylpyrrole-3-carboxylate)	2.5
Batrachotoxinin A-20-(2,4,5-trimethylpyrrole-3-carboxylate)	1
Batrachotoxinin A-20-(2,4-dimethyl-5-ethylpyrrole-3-carboxylate)	8
Batrachotoxinin A-20(*N*,2,4,5-tetramethylpyrrole-3-carboxylate)	280
Batrachotoxinin A-20-pyrrole-2-carboxylate	1000
Samandarine	300
Pumiliotoxin A	2500
Pumiliotoxin B	1500

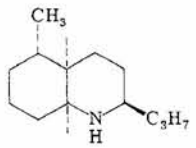
Batrachotoxin is an extremely active cardiotoxin that interferes with conduction in the heart and causes extrasystoles, ventricular fibrillation, and death. Blockage of neuromuscular transmission was observed in a variety of preparations (Märki and Witkop, 1963). Batrochotoxin does not affect the nerve action potential at concentrations which do block neuromuscular transmission. The poison is temperature-dependent with an optimum activity at 37°C. It first blocks muscle contraction induced by neural stimulation and later blocks contraction induced by direct stimulation of the muscle. A microelectrode study on the extensor digitorum muscle of the rat indicated an increase of miniature end plate potentials (mepp) from 6–8 to 600/second for the first 20 minutes after which their amplitude and frequency decreased. These effects, elicited by 1×10^{-8} *M* batrachotoxin, are similar to those caused by 5×10^{-4} *M* ouabain (strophantidin). Batrachotoxin does not affect the action potential generating mechanism of either muscle or nerve. It does not inhibit aectylcholine esterase nor does it inhibit the sodium/potassium-dependent ATPase of muscle. The effects of batrachotoxin, which are blocked by tetrodotoxin, appear due to the selective, irreversible increase in membrane permeability to sodium ions which is evoked by low concentrations of batrachotoxin. (Albuquerque *et al.*, 1970).

IV. ALKALOIDS FROM OTHER DENDROBATID FROGS

Extracts from a variety of other dendrobatid frogs (*Phyllobates vittatus, P. lugubris, Dendobrates auratus, D. histrionicus, D. pumilio, D. granuliferus, D. minutus, D. leucomelas, Colestethus talamancae, C. inquinalis, C. pratti,* and *C. nubicola*) from Costa Rica, Panama, Colombia, and Venezuela have been examined for steroidal alkaloids. Only *Phyllobates vittatus* (southwestern Costa Rica) and possibly the much smaller *Phyllobates lugubris* (Bocas del Toro, Panama) contained (homo)batrachotoxin and batrachotoxinin A. Basic extracts from frogs of the genus *Dendrobates* contained a different type of alkaloid. The basic extracts from frogs of the genus *Colostethus* were of low toxicity and contained small amounts of alkaloids of undetermined types.

The toxic principles from *Dendrobates pumilio* were isolated by thin-layer and column chromatography and analyzed by mass spectrometry (Daly and Myers, 1967). Three major alkaloids, pumiliotoxin A ($C_{19}H_{33}NO_2$, MLD 2.5 mg/kg mouse), pumiliotoxin B ($C_{19}H_{33}NO_3$, MLD 1.5 mg/kg mouse), and pumiliotoxin C ($C_{13}H_{25}N$) were present. Each of these frogs (*D. pumilio*) contains 50–500 μg of pumiliotoxin A and B. The structure of pumiliotoxin C (VIII) was determined by X-ray analysis of the crystalline carbonate

(J. W. Daly *et al.*, 1970). Pumiliotoxin A and B are closely related in structure but contain a 9-carbon side chain instead of the isopropyl group of pumiliotoxin C and one or more hydroxyl groups or double bonds.



(VIII)

Pumiliotoxin A and B have also been isolated from extracts derived from the Panamanian poison arrow frog (*Dendrobates auratus*), which contain, in addition, a number of other structurally related alkaloids. This general type of alkaloid showed mass spectra characterized by an intense fragment ion at m/e 168 or 152 ($C_{10}H_{16}NO$ or $C_{10}H_{18}N$) due to loss of the side chain.

Pumiliotoxin A or B, when injected subcutaneously in mice, cause locomotor difficulty and extensor movements of the hind limbs, followed by clonic convulsions and death.

V. SUMMARY

The investigation of pharmacologically active compounds contained in cutaneous secretions from various anurans has already led to the discovery of a number of compounds of novel structure such as dehydrobufotenine (II) (Märki *et al.*, 1961), spinaceamine (IV), samandarine (V), batrachotoxin (VIb), and pumiliotoxin C (VIII). Table I presents the results from some 170 anurans out of a total of approximately 2600 species; these data show that much more research is required. The steroidal alkaloid fraction, for example, has been investigated in only a few of these 170 species. Further chemical and pharmacological studies would be expected to lead to the discovery of other novel compounds, while biochemical studies on their biosynthesis and biological and biochemical studies on their function should allow further insight into the evolution of these compounds in amphibians.

ACKNOWLEDGMENT

The authors wish to thank Dr. Charles W. Myers of the American Museum of Natural History, for his invaluable advice during the preparation of this manuscript.

REFERENCES

Abel, J. J., and Macht, D. I. (1912). *J. Pharmacol. Exptl. Therap.* **3**, 319.

Albuquerque, E. X., Warnick, J. E., Daly, J., and Witkop, B. (1970). *Science* (in press).

Anastasi, A., Erspamer, V., and Bertaccini, G. (1965a). *Comp. Biochem. Physiol.* **14**, 43.

Anastasi, A., Erspamer, V., Bertaccini, G., and Cei, J. M. (1965b). *In* "Hypotensive Peptides" (Erdos, E. G., Back, N., and Sicuteri, F., eds.), p. 76. Springer-Verlag, New York.

Anastasi, A., Erspamer, V., and Endean, R. (1968). *Arch. Biochem. Biophys.* **125**, 57.

Bertaccini, G., Cei, J. M., and Erspamer, V. (1965). *Brit. J. Pharmacol.* **25**, 363.

Boulenger, G. A. (1913). *Proc. Zool. Soc., London* **1913**, 1029.

Breder, C. M., Jr. (1946). *Bull. Am. Mus. Nat. Hist.* **86**, 375.

Cei, J. M., and Erspamer, V. (1966). *Copeia* **1**, 74.

Cei, J. M., Erspamer, V., and Roseghini, M. (1967). *Syst. Zool.* **16**, 328.

Cei, J. M., Erspamer, V., and Roseghini, M. (1968). *Syst. Zool.* **17**, 219.

Chen, K. K., and Chen, A. L. (1934). *Arch. Intern. Pharmacodyn.* **47**, 297.

Chen, H. M., and Chen, K. K. (1951). *J. Pharmacol. Exptl. Therap.* **102**, 286.

Daly, J. W., Witkop, B., Bommer, P., and Biemann, K. (1965). *J. Am. Chem. Soc.* **87**, 124.

Daly, J. W., and Heatwole, H. (1966). *Experientia* **22**, 764.

Daly, J. W., and Myers, C. W. (1967). *Science* **156**, 970.

Daly, J. W., Tokuyama, T., Habermehl, G., Karle, I. L., and Witkop, B. (1969). *Ann. Chem.* **729**, 198.

DeCaro, G., Endean, R., Erspamer, V., and Roseghini, M. (1968). *Brit. J. Pharmacol. Chemotherap.* **33**, 48.

Erspamer, V. (1954). *Pharmacol. Rev.* **6**, 425.

Erspamer, V. (1959). *Arch. Biochem. Biophys.* **82**, 431.

Erspamer, V., Cei, J. M., and Roseghini, M. (1963). *Life Sciences* 825.

Erspamer, V., Anastasi, A., Bertaccini, G., and Cei, J. M. (1964a). *Experientia* **20**, 489.

Erspamer, V., Bertaccini, G., and Urakawa, N. (1964b). *Japan. J. Pharmacol.* **14**, 468.

Erspamer, V., Roseghini, M., and Cei, J. M. (1964c). *Biochem. Pharmacol.* **13**, 1083.

Erspamer, V., Vitali, T., Roseghini, M., and Cei, J. M. (1964d). *Arch. Biochem. Biophys.* **105**, 620.

Erspamer, V., and Anastasi, A. (1965). *In* "Hypotensive Peptides," (Erdös, E.G., Back, H., and Sicuteri, F., eds.), p. 63. Springer-Verlag, New York.

Erspamer, V., DeCaro, G., and Endean, R. (1966a). *Experientia* **22**, 738.

Erspamer, V., Roseghini, M., Endean, R., and Anastasi, A. (1966b). *Nature* **212**, 204.

Gessner, P. K., Godse, D. D., Krull, A. H., and McMullan, J. M. (1968). *Life Sciences* **7**, 267.

Habermehl, G. (1965). *Z. Naturforsch.* **20**, 1129.

Henderson, F. G., Welles, J. S., and Chen, K. K. (1960). *Proc. Soc. Exptl. Biol. Med.* **104**, 176.

Henderson, F. G., Welles, J. S., and Chen, K. K. (1962). *Science* **136**, 775.

Kiss, G., and Michl, H. (1962). *Toxicon* **1**, 33.

Lábler, L., Keilová, H., Sorm, F., Kornalik, F., and Styblová, A. (1968). *Toxicon* **5**, 247.

Latham, M. (1966). *Natl. Geographic* **129**, 682.

Lee, H. M., and Chen, K. K. (1951). *J. Pharmacol. Exptl. Therap.* **102**, 286.

Märki, F., Axelrod, J., and Witkop, B. (1962). *Biochim. Biophys. Acta* **58**, 367.

Märki, F., Robertson, A. V., and Witkop, B. (1961). *J. Am. Chem. Soc.* **83**, 3341.

Märki, F., and Witkop, B. (1963). *Experientia* **19**, 329.

Mezey, K. (1947). *Rev. Acad. Colombia Bogotá* **7**, 319.

Michl, H., and Kaiser, E. (1963). *Toxicon* **1**, 175.

Mosher, H. S., Fuhrman, F. A., Buchwald, H. D., and Fisher, H. G. (1964). *Science* **144**, 1100.
Nakajima, T. (1968). *Chem. Pharm. Bull.* **16**, 769, 2088.
Oestlund, E. (1954). *Acta Physiol. Scandanavia* **31**, Suppl. 112, 55.
Posada Arango, A. (1871). *Arch. Med. Nav.* **16**, 203.
Santessen, C. G. (1935). *Ethnological Studies* **1**, 105.
Tokuyama, T., Daly, J. W., Witkop, B., Karle, I. L., and Karle, J. (1968). *J. Am. Chem. Soc.* **90**, 1917.
Tokuyama, T., Daly, J. W., and Witkop, B. (1969). *J. Am. Chem. Soc.* **91**, 3931.
Welsh, J. H., and Zipf, J. B. (1966). *J. Cell. Physiol.* **68**, 25.
Welsh, J. H. (1964). "Comparative Neurochemistry" (D. Richter, ed.), p. 355. Pergamon Press, London.