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The distribution of various amines and polypeptides of the skin in 90 species of five frog families show general agreement with classifications based on other criteria. In particular, the skin biochemistry generally supports the subgeneric divisions of *Leptodactylus* based on secondary sex characters; the isolated position of *Odontophrynus* among the Ceratophryidae indicated by serological evidence is supported; *Phyllomedusa* differs strongly from other hylid genera in its possession of large amounts of polypeptides; and the presence of bufotenine in *Melanophryniscus* confirms the assignment of the genus to the Bufonidae. There is great variation within and between species of *Bufo* in amounts of amines of the skin.

INTRODUCTION

ATTENTION has been focused in the past decade on comparative biochemistry as capable of offering keys to the solution of problems of evolution and classification as well as elucidating problems of general biochemistry. Wittliff, for example, used amines and other skin secretions of toads to distinguish species and species hybrids (Wittliff, 1962, 1964). For the past four years we have been carrying out a systematic study of amines and polypeptides present in skins of amphibians of South and Central America. Results of this survey appear to have important taxonomic implications as well as being of biochemical and pharmacological interest (identification of at least five new pharmacologically active polypeptides and about 10 new naturally occurring amines).

The different anuran groups represent exceptional material for biochemical comparisons because of the large number of species included. Data include analyses of indole-, phenyl-, and imidazolealkylamines; polypeptides studied are principally those which have physiological effects upon smooth muscle preparations. Sex differences or seasonal variation were not evidenced at the present stage of our comparative researches.

MATERIALS AND METHODS

Animals examined included 90 species distributed among five families of neotropical anurans: Leptodactylidae, Ceratophryidae, Hylidae, Bufonidae, and Ranidae.

Collection and extraction of the skins.—The skins, removed from the animals immediately after sacrifice, were either extracted in loco with three to four parts of methanol (w/v) or spread out and dried in the shade. Dried skins were extracted in the laboratory with eight to 10 times their weight of 70% acetone and 80% methanol. Methanol was necessary to extract active polypeptides; generally acetone was best for the extraction of the alkylamines since it dissolves less inert material. However, the best procedure for extracting specific active compounds had to be established in each case by experiment. Skins were soaked for a week in the solvent, the extract was decanted, and the skins were reextracted for another week with the same solvent. Extracts were pooled, filtered, and stored in a refrigerator in dark bottles. Such crude extracts do not lose activity if stored for months or even years.

Chromatographic analysis.—After evaporating the solvent and defatting with petroleum ether, extracts were chromatographed on Whatman No. 1 paper, using an ascending technique. Solvents employed were *n*-butanol: acetic acid : water (40 : 10 : 50) and *n*-butanol : 30% methylamine (80 : 30). Chromatograms were developed with the following reagents: diazotized sulphanilic acid (Pauly reagent), diazotized *p*-nitroaniline, Heinrich and Schuler's NNCD reagent (2-chloro-4-nitro-1-diazobenzene-α-naphthalene sulphuric acid), dichloroquinone-chlorimide reagent (Gibbs' reagent), *p*-dimethylaminobenzaldehyde reagent, Folin reagent for amino acids (1, 2-naphthoquinone-4-sulphonic sodium salt), and ninhydrin reagent. Crude estimates of the concentration of constituents in extracts were made by visual comparison of the intensity of spots on developed chromatograms with spots produced by known amounts of corresponding synthetic compounds. For quantitative estimations, fractions were eluted from chromatograms into test tubes. Fractions and standards were treated with appropriate reagents and color intensities compared in a spectrophotometer.

Bioassay.—Biological activity of all the extracts was assayed on the following preparations: rabbit large intestine, rat large intestine, rat estrous uterus, guinea pig ileum, frog rectus abdominis muscle. Effects on blood pressure and respiration were assayed using dogs. Diuretic effects were measured on the rat. Earlier reports (Erspamer, 1959a, 1959b; Anastasi and Erspamer, 1963) cited details of procedures employed in purification, isolation, and identification of biologically active substances discovered during this survey.

Standards.—The following synthetic compounds were available for chromatographic and bioassay standards: 5-hydroxytryptamine creatinine sulfate (5-HT), N-methyl-5-HT creatinine sulfate, bufotenine base, dehydrobufotenine base, 5-hydroxyindoleacetic acid, tryptamine hydrochloride, indole acetic acid, tryptophan, 5-hydroxytryptophan, *p*-tyramine, *m*-tyramine, *p*-tyrosine, *m*-tyrosine, *o*-tyrosine, candicine iodide, leptodactyline picrate, histamine dihydrochloride, N-methylhistamine dihydrochloride, N,N-dimethylhistamine dihydrochloride, spinaceamine dihydrochloride, 6-methylspinaceamine dihydrochloride, histidine, bradykinin (Sandoz), eleidosin (Farmitalia), hypertensin (CIBA) and vasopressin (Sandoz).

Results and Discussion

Leptodactylidae.—Skin extracts of primitive Leptodactylidae either lack or contain only scanty amounts of biologically active polypeptides and amines. Genera examined include *Elosia* (Elosiinae), *Calyptocephalella* (Calyptocephalellinae), *Eleutherodactylus* (Eleutherodactylinae), *Telmatobius* and *Batrachophrynus* (Telmatobiinae), and *Thoropa*, *Cycloramphus*, and *Eupsophus*. Maximum values of leptodactyline and 5-HT occurred in *Calyptocephalella gayi* and *Thoropa miliaris* (10–12 μg/g of dry skin).

The Leptodactylinae include species which virtually lack and others which contain high concentrations of active polypeptides and amines. The genus *Pleurodema*, for example, contains little leptodactyline (2–25 μg/g of dry skin), occasionally 5-HT (200–250 μg/g in the coxal glands of *Pleurodema bufonina*), but lacks biologically active polypeptides. *Physalaemus*, while virtually lacking amines, contains large amounts of physalaemin, an eleidosin-like polypeptide, which potently lowers blood pressure and stimulates extravascular smooth muscle (Erspamer, Bertaccini, and Cei, 1962).

Skin of true *Leptodactylus* lacks polypeptides but contains a formidable array of amines (Table 1). The spectrum of amines present in different species is in fair accordance with the scheme of subgeneric division suggested by Miranda–Ribeiro (1926) and Lutz (1930). The Cavicola species group (i.e., *Leptodactylus prognathus*, *L. bufonius*, *L. sibilatrix*) lacks amines; the Platimantis group (*L. podicipinus*, *L. melanonotus*) contains large amounts of leptodactyline and indolealkylamines. Using amine content of their skins, the Pachypus species group may be differentiated into two groups in full accordance with morphological characteristics such as the stout forearm and sex characters. One group, represented by *L. ocellatus*, *L. chaquensis*, and *L. bolivianus*, is rich in phenylalkylamines (up to 9,000 μg leptodactyline base per g of dry tissue), but lacks indolealkylamines and imidazolealkylamines. The second group, represented by *L. pentadactylus* and *L. laticeps*, both characterized by peculiar horny pads on the chest of the males, is poor in phenylalkylamines but rich in indolealkylamines and, what is more unusual, in imidazolealkylamines. These include, not only widely distributed histamine, but also the less common and poorly known

TABLE 1. CONTENT OF AMINES (μg free bases/g dry skin) IN *Leptodactylus*.

Groups and species[1]	Indolealkylamines[2]		Phenylalkylamines[2]		Imidazolealkylamines[2]					Other amines
	5-HT	Bufotenidine[3]	Candicine	Leptodactyline	Histamine	N-Methyl-histamine	N,N-Dimethyl-histamine	Spinace-amine	6-Methyl-spinaceamine	
"Cavicola"										
L. bufonius—1	0	0	0	4–5	0	0	0	0	0	
L. prognathus—2	0	0	0	3–4	0	0	0	0	0	
"Platimantis"										
L. podicipinus					0	0	0	0	0	
podicipinus—3	640	15–20	0	3,100–5,300	0	0	0	0	0	
petersi—4	1–1.5	2.5–3	0	750	0	0	0	0	0	N-Methyl-5-HT 10
L. melanonotus—5	35	25	0	265						
"Pachypus"										
L. pentadactylus										
labyrinthicus, 1961, Sept.—6	1,900	0	0	12.5	740	670	210–240	120	400–425	
labyrinthicus, 1961, Feb.—6	200	0	0	1.5	100	100–120	30–40	20	80–100	
pentadactylus—7	140	0	40–50	9.0	10–20	0	0	0	0	Tyramine <5
dengleri—8	50–65	600	2–3 (?)	11–14	35	0	0	0	0	
L. laticeps—9	280	0	0	2–3	260–280	0	0	5–10 (?)	0	
L. ocellatus—10	0	0	0	180–8,800	0	0	0	0	0	
L. chaquensis—11	0	0	0	55–530	0	0	0	0	0	
L. bolivianus—12	0	0	0	480	0	0	0	0	0	
L. rubido—13	7	40–45	0	200	0	0	0	0	0	

[1] Localities: 1, 2, 3, 10, 11 from Argentina; 6 from Misiones, Argentina; 4, 13 from Tingo Maria, Peru; 9 from Chaco, Argentina; 5 from Mexico; 7, 12 from Iquitos, Peru; 8 from Costa Rica.

[2] 0 = nondetectable ($<$ 1–2 μg/g tissue).

[3] Expressed as bufotenine.

TABLE 2. CONTENT OF INDOLEALKYLAMINES (μg of free bases/g dry skin) IN SOUTH AMERICAN *Bufo*.[1]

Bufo species	5-HT	N-Methyl-5-HT	Bufotenine	Bufotenidine	Dehydrobufotenine	Bufothionine
B. marinus						
m. marinus						
Surinam						
Paramaribo I)	100	130	4	0	3,000	350–400
II)	135	100	n.d.	0	6,000	15–30
Costa Rica	100	50	n.d.	0	2,200	260
m. poeppigi						
Bolivia						
(Coroico)	150–180	120–170	n.d.	n.d.	1,100	230
Peru						
(Tingo Maria)	150–170	80–120	n.d.	n.d.	270	230
B. ictericus						
Brasil (Rio)	10–20	10–20	n.d.	0	4,500	250
Argentina						
(Misiones)	50–70	40–60	15	0	3,300	375
B. paracnemis						
Argentina						
(Formosa)	70	80	4,300	180	180	290
(Tucuman)	25	0	2,800	220	120	110
(Corrientes)	10–20	0	5,000	650	250	300
(Laguna Ibera)	35	0	4,500	400	270	300
(Misiones)	100	200	2,500	250	1,500	360
B. arenarum						
Argentina						
(Tucuman)	60–600	60–400	1,100–2,600	40–60	550–700	250–450
(Cordoba)	40–120	30–100	800–1,600	50	230–600	70–170
(Mendoza)	35–75	0	1,200–2,100	65–130	350–600	220–450
B. spinulosus						
s. spinulosus						
Peru, Cuzco	n.d.	n.d.	220–250	30	180	830
s. arunco						
Chile, Santiago	n.d.	n.d.	15–20		230	360
s. trifolius						
Peru	n.d.	n.d.	500–600	100	n.d.	n.d.
B. granulosus						
g. major						
Argentina-Formosa	60	20–25	140	0	n.d.	300
Chaco-						
Resistencia-*S*[2]	250	130	200	0	550	780
g. fernandezae						
Argentina						
Chaco-						
Resistencia-*S*[2]	150	500	150	0	2,400	1,150
Buenos Aires	60	330	150	0	1,200	900

[1] n.d. = not detectable.
[2] S = sympatric populations.

N-methylhistamine and N,N-dimethylhistamine, and the hitherto unknown spinaceamine and 6-methylspinaceamine. Further, a clear-cut biochemical distinction can be made between three subspecies of *L. pentadactylus* (Table 1).

Ceratophryidae.—Skins of this peculiar neotropical stock of anurans are very poor in indolealkylamines, with the exception of the genus *Odontophrynus* (*O. americanus, O. occidentalis*: 5-HT, 310 to 2,600 $\mu g/g$ of dry skin). Serologically this genus lies far away from other studied representatives of this ancient anuran branch (Cei, 1965).

Hylidae.—Among the Hylidae, the genera *Hyla, Sphoenorhynchus, Osteocephalus, Trachycephalus, Corythomantis,* probably *Triprion,* and *Gastrotheca,* lack or are poor in amines and polypeptides. However, species of the highly specialized genus *Phyllomedusa* (*Phyllomedusa sauvagii, P. hypochondrialis, P. annae, P. rohdei, P. burmeisteri, P. callidryas, P. dacnicolor*) contain large amounts of powerful bradykinin-like and physalaemin-like polypeptides. To date, at least five active polypeptides have been identified and it seems possible that each species of *Phyllomedusa* has a characteristic polypeptide spectrum. A strikingly specialized position of *Phyllomedusa* as an independent hylid branch is suggested by this material (cf. Cei, 1963).

Bufonidae.—Skins of *Bufo* lack biologically active polypeptides, but contain phenylalkylamines and large quantities of a variety of indolealkylamines (Table 2). Marked differences in the spectrum of indolealkylamines occur among different species, and occasionally even between populations from different geographic areas. *Melanophryniscus moreirae* skin contains a new hydroxyphenylalkylamine, as well as considerable bufotenine (more than 2,000 $\mu g/g$ of dry skin). The presence of bufotenine confirms the relationship of *Melanophryniscus* with other bufonid genera as suggested, on a morphological basis, by Griffiths (1959) and other herpetologists.

Ranidae.—The few species of Central and South American Ranidae examined were similar to European and Japanese Ranidae in that their skins also contained indolealkylamines (5-HT) and bradykinin-like polypeptides.

Conclusions

It appears as if the amines and polypeptides present in amphibian skin offer valuable biochemical evidence for determining the positions of suprageneric taxa, and also for the solution of controversial problems concerning interspecific and subspecific relationships. As yet it is not possible to state the adaptive and biological significance of these pharmacologically active compounds.

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