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INDOLE-, IMIDAZOLE-, AND PHENYL-ALKYLAMINES IN THE SKIN OF THIRTEEN *LEPTODACTYLUS* SPECIES

V. ERSPAMER, M. ROSEGHINI and J. M. CEI

Institute of Pharmacology, University of Parma, Parma, Italy
and
Institute of Biology, National University of Cuyo, Mendoza, Argentina

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Abstract—The skin of thirteen *Leptodactylus* species and sub-species has been examined in regard to its content in biogenic amines. This tissue presents, especially in *Leptodactylus pentadactylus labyrinthicus* and some other species, an unusually rich miscellany of amines, including at least two indolealkylamines (5-hydroxytryptamine and bufotenidine), three hydroxyphenylalkylamines (*p*-tyramine, candicine, leptodactyline) and five imidazolealkylamines (histamine, N-methylhistamine, N,N-dimethylhistamine, spinaceamine and 6-methylspinaceamine). It is concluded that the *Leptodactylus* skin must possess aromatic acid decarboxylase activity, tryptophan-5-hydroxylase activity and N-methyltransferase activity. The skin of nearly every *Leptodactylus* species and sub-species is characterized by a particular spectrum of amines, which evidently may help in systematics of these species.

IT HAS been shown in other papers[1,3,4] that the skin of different species and sub-species of *Leptodactylus*, an amphibian genus living in tropical America, may contain considerable amounts of leptodactyline, candicine and imidazolealkylamines.

This communication gives a more complete account of the occurrence of the above amines as well as of other biogenic amines in the skin of the thirteen *Leptodactylus* species examined so far.

It will appear that results are not only of obvious biochemical interest, but also of considerable importance in zoological taxonomy.

METHODS AND MATERIALS

Synthetic compounds.

The following synthetic compounds were available for comparison: 5-hydroxytryptamine creatinine sulfate (5-HT, 0·43); bufotenine base; tyramine. HCl (0·74); candicine iodide (0·64); leptodactyline picrate (0·44); histamine. 2HCl (0·6); N′-methylhistamine. 2HCl (0·63); N′,N′-dimethylhistamine. 2HCl (0·66); spinaceamine. 2HCl.H_2O (0·57); 6-methylspinaceamine. 2HCl (0·66). In parentheses are the equivalents in free bases.

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Amphibian material

Leptodactylus ocellatus: more than 100 adult specimens captured in 1961–1963 in different places in Argentina. Average weight of a dry skin: 1·5 g.

Leptodactylus chaquensis: more than 200 adult specimens collected as above. Weight of a dry skin: 0·6–1·8 g.

Leptodactylus bolivianus: 1 specimen, captured at Iquitos, Peruvian Amazonia, November 1962. Weight of the dry skin = 2 g.

Leptodactylus podicipinus podicipinus: 56 adult specimens captured at Corrientes, Argentina, October 1962. Weight of a dry skin = 0·1 g.

Leptodactylus podicipinus petersi: 5 specimens captured at Tingo Maria, Peru, February 1963. Weight of a dry skin = 0·3 g.

Leptodactylus rubido cope: 2 specimens captured at Tingo Maria, Peru, February 1963. Weight of a dry skin = 1·25 g.

Leptodactylus melanonotus: 35 specimens captured at Vera Cruz, Mexico, October 1963. Weight of a dry skin = 0·02 g.

Leptodactylus bufonius: 21 specimens captured at Tucuman, Argentina, March 1960. Weight of a dry skin = 0·25 g.

Leptodactylus prognatus: 78 specimens captured at Tucuman, Argentina March 1959. Weight of a dry skin = 0·06 g.

Leptodactylus pentadactylus labyrinthicus: 7 adult specimens, captured in Misiones, Argentina, February 1961 (weight of a dry skin = 10·4 g) and 5 adult specimens captured at the same place, September 1961 (weight of a dry skin = 6·4 g).

Leptodactylus pentadactylus pentadactylus: 5 adult specimens captured at Iquitos, Peruvian Amazonia, September 1962. Weight of a dry skin = 10·4 g.

Leptodactylus pentadactylus dengleri: 1 adult specimen, captured in Costarica, May 1962. Weight of the dry skin = 5·4 g.

Leptodactylus laticeps: 1 adult female specimen captured in Misiones, Argentina, February 1961. Weight of the dry skin = 5·6 g.

The fresh skins were carefully spread out and dried in the shade.

Extraction procedure

Immediately after their arrival in Italy by air mail the dried skins were minced with scissors and then immersed in 8 parts (w/v) of 70% acetone or, less frequently, 80% methanol. The liquid was decanted after 4–7 days, and the skins were re-extracted for other 5–7 days with 5–6 parts of the same solvent. The extracts, yellow or brown yellow in colour, were combined and filtered. Kept in dark bottles and refrigerated they may be stored for a long time, for months or even years, without appreciable losses in biogenic amines.

Paper chromatography

The ascending unidimensional technique on Whatman No. 1 paper was routinely employed. Chromatograms were run at 18° for 20–30 hr, using as a rule the *n*-butanol–acetic acid–water (40:10:50) and the *n*-butanol–30% methylamine (80:30) solvent mixtures. In the separation and paper-chromatographic estimation of imidazole-alkylamines other solvent systems were also profitably used: 1-pentanol–pyridine–water–30% methylamine (40:40:10:1), and methylethylketone–pyridine–water–30% methylamine (65:15:10:0·5).

The spraying reagents used for the detection and identification of the different compounds were as follows: (a) aqueous solution of diazotized sulfanilic acid (Pauly reagent) followed by 3–5% aqueous sodium carbonate; (b) aqueous solution of diazotized *p*-nitroaniline followed by sodium carbonate; (c) 0·1–0·3% solution of Heinrich and Schuler NNCD reagent (2-chloro-4-nitro-1-diazobenzene-α-naphthalene sulfuric acid) in 0·1 M HCl; (d) 0·3% solution in 50% ethanol of Echtrotsalz B Fluka (diazotized 1-amino-2-methoxy-4-nitrobenzene) followed by sodium carbonate; (e) 0·05–0·1% alcoholic solution of dichloroquinone chlorimide (Gibbs reagent) followed by sodium carbonate; (f) 1–2% alcoholic solution of *p*-dimethylamino-benzaldehyde, followed by exposure of the chromatograms to HCl vapours in a glass cabinet; (g) 1% alcoholic solution of Folin reagent for aminoacids (1,2-naphtho-quinone-4-sulphonic sodium salt) followed by sodium carbonate; (h) diluted Folin-Ciocalteu reagent, followed by sodium carbonate or exposure to ammonia vapours.

As stated in another paper,[4] imidazole derivatives are characterized by the positivity of reactions (a), (b), and eventually (g) and by the negativity of all other reactions; phenolic derivatives by the positivity of the same reactions and, in addition, of reaction (h) and eventually (e); 5-hydroxyindole compounds by the positivity of all the tested reactions.

Semi-quantitative estimation of a given natural compound on paper chromatograms was carried out by visual comparison of spots produced by different amounts of crude or semi-purified skin extracts with spots produced by different known amounts of the corresponding synthetic compound.

Bioassay

Quantitative estimation of 5-HT was carried out on the atropinized oestrous uterus of the rat; estimation of biologically active imidazolealkylamines on the guinea-pig ileum suspended in Krebs solution containing atropine and 2-bromolysergic acid dietylamide (BOL) 10^{-7}; finally, estimation of leptodactyline was carried out on the frog rectus abdominis muscle.

RESULTS

Leptodactylus pentadactylus

The three examined *Leptodactylus pentadactylus* sub-species have given the most interesting results.

Figure 1 gives a schematic representation of paper chromatograms obtained with ethanol eluates from an alkaline alumina column loaded with a crude acetone extract of the skin of *Leptodactylus pentadactylus labyrinthicus*. The figure represents all the spots developed, of course on different chromatograms, with the Pauly reagent, the NNCD reagent, the Gibbs reagent and the *p*-dimethylaminobenzaldehyde reagent.

Figures 2 and 3 refer to *Leptodactylus pentadactylus pentadactylus* and to *Leptodactylus pentadactylus dengleri*, respectively. They are again composite chromatograms, showing all the spots developed with the four above spraying reagents.

The R_f values of the different spots in the *n*-butanol–acetic acid–water mixture may be inferred from the accompanying figures. Additional data concerning other solvent systems are reported in previous papers.[1, 3, 4]

Table 1 shows the colour reactions given by the most important spots with four spraying reagents.

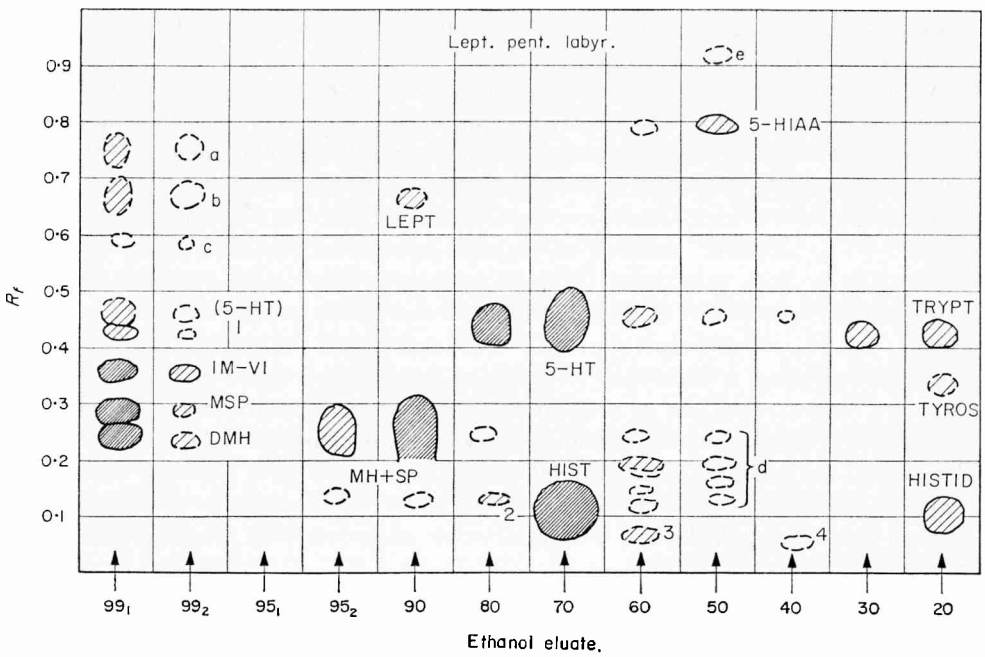


FIG. 1. Paper chromatograms of the ethanol eluates obtained from an alumina column loaded with the skin extract of *Leptodactylus pentadactylus labyrinthicus* Sept. 1961. Solvent, *n*-butanol–acetic acid–water; developing reagents, NNCD reagent + Pauly reagent + Gibbs reagent + *p*-dimethylaminobenzaldehyde reagent. Amounts of eluates corresponding to 0·1 g of dry skin were applied on paper at arrows.

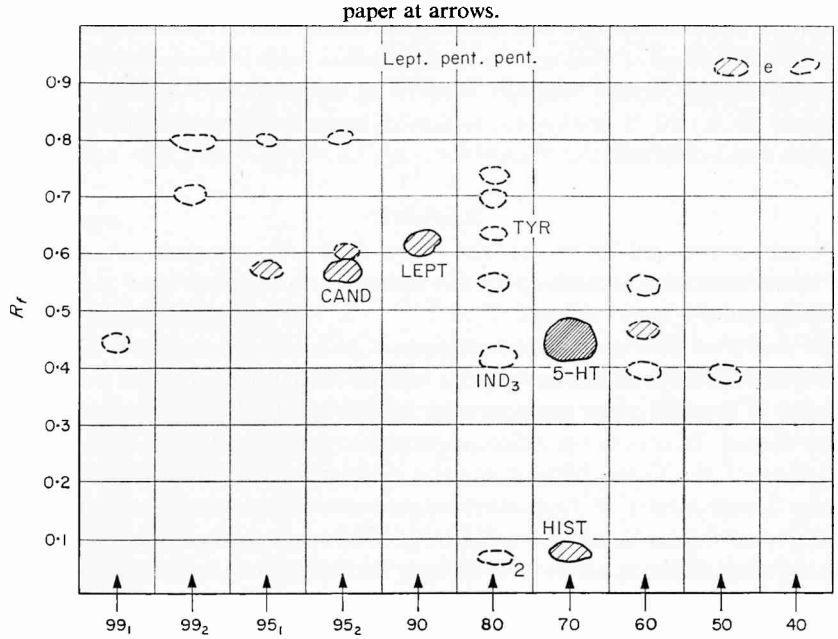


FIG. 2. Paper chromatograms of the ethanol eluates obtained from an alumina column loaded with the skin extract of *Leptodactylus pentadactylus pentadactylus*. Solvent and developing reagents as described in the text of Fig. 1. Amounts of eluates corresponding to 0·25 g of dry skin were applied on paper at arrows.

A number of spots could easily be identified by their R_f values and colour reactions, by superimposition experiments with synthetic substances and by bioasssay, with histamine (HIST), N-methylhistamine (MH), N,N-dimethylhistamine (DMH), spinaceamine (SP), 6-methylspinaceamine (MSP), 5-hydroxytryptamine (5-HT), bufotenindine (BUFOT), *p*-tyramine (TYR), leptodactyline (LEPT), candicine (CAND), 5-hydroxyindoleacetic acid (5-HIAA), tryptophan (TRYPT), *p*-tyrosine (TYROS), and histidine (HISTID), respectively.

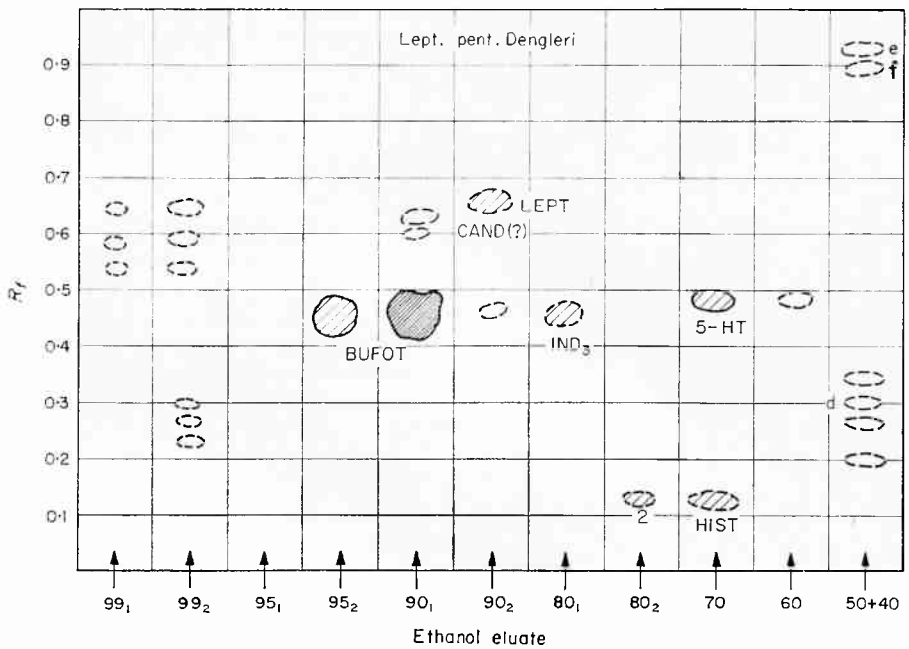


FIG. 3. Paper chromatograms of the ethanol eluates obtained from an alumina column loaded with the skin extract of *Leptodactylus pentadactylus dengleri*. Solvent and developing reagents as described in the text of Fig. 1. Amounts of eluates corresponding to 0·2 g of dry skin were applied on paper at arrows.

The chemical structure of the compounds constituting the other spots is unknown. However, it may be suggested, on the basis of their colour reactions, that spots IM–VI, 1, 2, 3 and 4 are of imidazolic, spots a, b, c, d and e of indolic, and spot f of phenolic nature. The possibility cannot be ruled out that some of the minor spots are artifacts, constituted by alteration products appearing during drying of the skin, or during preparation or chromatography of the extracts.

Particular attention seems to be deserved by IND$_3$ spot which is made up by a 5-hydroxyindole derivative possessing the unusual characteristic of giving a negative or very faint reaction with *p*-dimethylaminobenzaldehyde. Erspamer and Bertaccini[2]

have found similar compounds in the urine of rats treated with 5-hydroxytryptophan and they have suggested that these indole derivatives might be substituted at the position 1, the presence of a free =NH group in the nucleus being apparently necessary for the appearance of the *p*-dimethylaminobenzaldehyde reaction.

TABLE 1. COLOUR REACTIONS OF THE INDOLIC, IMIDAZOLIC AND PHENOLIC COMPOUNDS OCCURRING IN SKIN EXTRACTS OF *Leptodactylus pentadactylus*

5-HT, 5-hydroxytryptamine; BUFOT, bufotenidine; 5-HIAA, 5-hydroxyindoleacetic acid; TRYPT, tryptophan; HIST, histamine; MH, N-methylhistamine; DMH, N,N-dimethylhistamine; SP, spinaceamine; MSP, 6-methylspinaceamine; HISTID, histidine; TYR, *p*-tyramine; CAND, candicine; LEPT, leptodactyline, TYROS, *p*-tyrosine

	NNCD reagent	Pauly reagent	Gibbs reagent	*p*-Dimethylamino-benzaldehyde reagent
Indoles				
5-HT	peach red	wine red	blue violet	blue
BUFOT	peach red	wine red	blue violet	blue
5-HIAA	peach red	wine red	blue violet	blue
TRYPT	orange yellow	0	0	violet
IND_3	wine red	wine red	blue violet	?
a	yellow	rose	blue violet	rose violet
b	peach red	wine red	blue	blue
c	peach red	wine red	blue violet	blue
d	orange	0	0	violet
Imidazoles				
HIST	0	pink red	0	0
MH	0	pink red	0	0
DMH	0	pink red	0	0
SP	0	orange yellow turning into orange red	0	0
MSP	0	orange yellow turning into orange red	0	0
HISTID	0	pink red	0	0
IM-VI	0	pink red	0	0
1, 2, 3, 4	0	pink red	0	0
Phenoles				
TYR	0	red	0	0
TYROS	0	red	0	0
CAND	0	red	0	0
LEPT	0	yellow	sky blue	0
e	0	yellow	sky blue	0
f	0	wine red	0	0

0 = negative reaction

Table 2 summarizes all the quantitative data on the content of indole-, imidazole- and phenyl-alkylamines in the *Leptodactylus pentadactylus* skin collected in this and in previous studies.

When transferred to the fresh skin, the above values should be divided by 3 or 4. In fact, 1 g of dry *Leptodactylus* skin corresponds to 3–4 g of fresh tissue.

TABLE 2. THE CONTENT OF BIOGENIC AMINES (IN μg FREE BASES PER g DRY TISSUE) IN THE SKIN OF THIRTEEN *Leptodactylus* SPECIES

Leptodactylus species	Indolealkylamines		Phenylalkylamines		Imidazolealkylamines					Other amines
	5-HT	Bufotenidine*	Candicine	Leptodactyline	Histamine	N-Methyl-histamine	N,N-Dimethyl-histamine	Spinaceamine	6-Methyl-spinaceamine	
L. pentadact. labyrinthicus										
Sept. 1961	1900	0	0	12·5	740	670	210–240	120	400–425	IM-VI 135-160
Feb. 1961	200	0	0	1·5	100	100–120	30–40	20	80–100	
L. pentadact. pentadactylus	140	0	40–50	9	10–20	0	0	0	0	Tyramine <5
L. pentadact. dengleri	50-65	600	2-3(?)	11–14	35	0	0	0		
L. laticeps	280	0	0	2–3	260–280	0	0	5–10(?)	0	IM-VI > 150
L. podicipinus podicipinus	640	15–20	0	3100–5300	0	0	0	0	0	
L. podicipinus petersi	1–1·5	2·5–3	0	750	0	0	0	0	0	
L. rubido cope	7	40–45	0	200	0	0	0	0	0	
L. melanonotus	35	25	0	265	0	0	0	0	0	N-Methyl-5-HT 10
L. ocellatus	0	0	0	180–8800	0	0	0	0	0	
L. chaquensis	0	0	0	55–530	0	0	0	0	0	
L. bolivianus	0	0	0	480	0	0	0	0	0	
L. bufonius	0	0	0	4–5	0	0	0	0	0	
L. prognatus	0	0	0	3–4	0	0	0	0	0	

0 = not-detectable (<1–2 μg/g tissue)
* expressed as bufotenine

Leptodactylus laticeps

Indole, imidazole, and phenolic derivatives occurring in skin extracts of *Leptodactylus laticeps* are essentially shown in Fig. 4. Quantitative data on 5-HT and histamine are reported in Table 2.

Spot IM-VI is certainly, as already stated, of imidazole nature and the elucidation of its structure is in progress. Substances constituting spots a and b are probably 5-hydroxyindole derivatives and substances constituting spots d indole derivatives lacking the —OH group on the indole ring (orange-yellow colour with NNCD, violet colour with *p*-dimethylaminobenzaldehyde).

It is of interest that *Leptodactylus laticeps* skin does not contain detectable amounts of either N-methyl- or N,N-dimethyl-histamine, while presenting exceptionally large amounts of compound IM-VI.

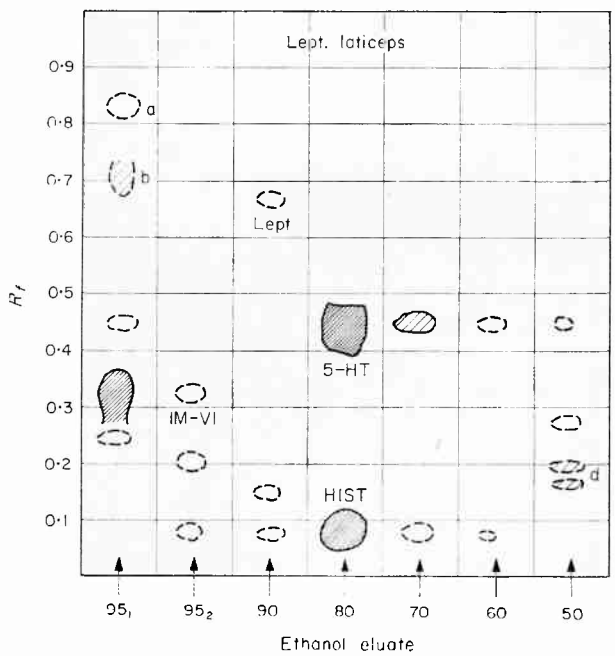


FIG. 4. Paper chromatograms of the ethanol eluates obtained from an alumina column loaded with the skin extract of *Leptodactylus laticeps*. Solvent and developing reagents as described in the text of Fig. 1. Amounts of eluates corresponding to 0·25 g of dry skin were applied on paper at arrows.

Leptodactylus podicipinus

The skin of the two *Leptodactylus podicipinus* sub-species considered in this study contains large amounts of leptodactyline and variable amounts of 5-HT and bufotenidine (Table 2).

Chromatograms of skin extracts of *Lept. podicipinus podicipinus* run in the butanol: acetic acid: water mixture show the following additional minor spots, made up of unknown substances: an indolic spot, corresponding to spot IND_3 of Fig. 3, in ethanol eluate 80_2; two phenolic spots in ethanol eluate 50, having the R_f values of 0·54 and 0·86, and giving red and yellow colour shades, respectively, with the Pauly reagent.

Similar chromatograms of *Lept. podicipinus petersi* show an imidazole spot in ethanol eluates 70 and 60 (R_f 0·05), and a spot of unknown nature (phenolic?) in ethanol eluates 50 and 40. This spot, having a R_f of 0·27, gives an orange colour with the Pauly reagent, and a light blue-grey colour with the Gibbs reagent.

Leptodactylus rubido cope

Skin extracts of *Leptodactylus rubido* contain leptodactyline, 5-HT and bufotenidine.

Chromatograms run with butanol–acetic acid–water show the following additional minor spots: a Pauly-positive spot with R_f 0·59 in ethanol eluates 80 and 70, a Pauly-positive spot with R_f 0·04 in eluates 70 and 60, two Gibbs-positive spots (sky-blue colour) in ethanol eluates 60 (R_f 0·34) and 50 + 40 (R_f 0·92), and finally three NNCD-positive spots (orange-yellow colour) with R_f values of 0·1, 0·12 and 0·17, respectively, in ethanol eluate 40.

Leptodactylus ocellatus, L. bolivianus and L. chaquensis

The only known biogenic amine contained in skin extracts of these three *Leptodactylus* species is leptodactyline (Table 2). Some unknown Pauly-positive and Gibbs-positive compounds have not been investigated further.

Leptodactylus bufonius and L. prognatus

Skin extracts of these last two *Leptodactylus* species are characterized by their content of very small amounts of leptodactyline and lack of detectable quantities of other known amines.

DISCUSSION

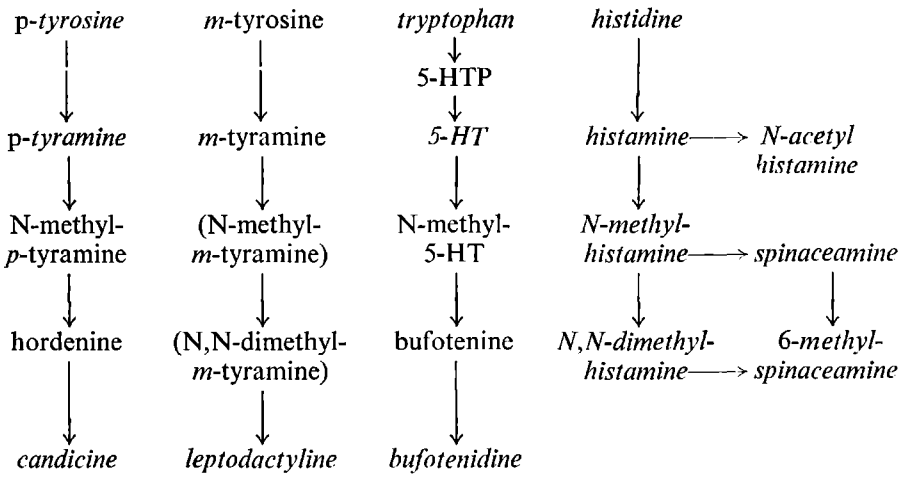
Results presented in this paper are of interest both from a general biochemical point of view and from the point of view of biochemical taxonomy.

It has been found that the skin of *Leptodactylus* may contain representatives of all the three classes of biogenic amines considered in the present study. Imidazolealkylamines are represented by at least five individual members, hydroxyphenylalkylamines by three members and 5-hydroxyindolealkylamines by two members.

The precursor aminoacid of all imidazolealkylamines may be considered histidine which is present in all skin extracts. Similarly present is *p*-tyrosine, the precursor of *p*-tyramine and candicine, and tryptophan, the remote precursor of 5-HT and bufotenidine. So far, search for 5-HTP, the immediate precursor of the 5-hydroxyindolealkylamines, and for *m*-tyrosine, the probable precursor of leptodactyline, has been unsuccessful.

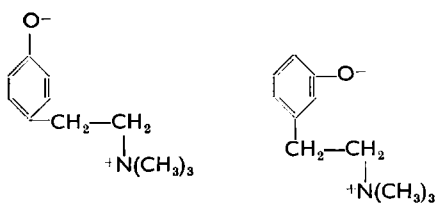
From our data it clearly appears that amines occurring in the *Leptodactylus* skin are most frequently represented by primary amines and quaternary ammonium bases, more rarely by secondary and tertiary amines.

The possible biochemical correlations existing between the different amines are summarized below.



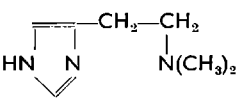
Compounds present in the *Leptodactylus* skin are in italics; compounds as yet not found in nature are in parentheses.

For the sake of clearness it seems opportune to report the chemical structure of the less familiar of the above compounds.

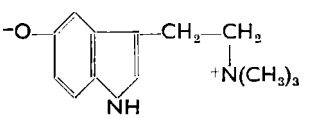


Candicine

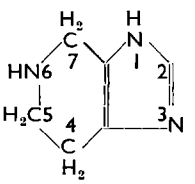
Leptodactyline



N,N-Dimethylhistamine



Bufotenidine



Spinaceamine

From the above it must be concluded that amphibian skin possesses L-aromatic acid decarboxylase activity, tryptophan 5-hydroxylase activity and an apparently strong N-methyl transferase activity. Although keeping in mind that static estimation of the content in amines of a tissue does not offer any reliable information on the turnover rate of these amines, it may reasonably be inferred that primary amines once formed in the *Leptodactylus* skin may be converted into their corresponding quaternary ammonium derivatives with considerable ease. This is shown by the not infrequent lack or scarcity of the primary amine in the presence of enormous amounts of the quaternary base. A typical example is that of the leptodactyline.

It is not easy to give a satisfactory explanation of the apparent differences in the spectrum of N-methyl transferase activity observed in the skin of different *Leptodactylus* species. The skin of *Leptodactylus pentadactylus labyrinthicus*, for example, is capable of methylating *m*-tryamine and histamine but not 5-HT; that of *Leptodactylus pentadactylus pentadactylus* *m*-tyramine and *p*-tyramine but again not 5-HT; finally, that of *Leptodactylus pentadactylus dengleri* does methylate both *m*-tyramine and 5-HT.

One is tempted to conclude from these data that *m*-tyramine is the best substrate for N-methyl transferase in the *Leptodactylus* skin, followed by *p*-tyramine, histamine and then 5-HT.

It has been emphasized that the results of the present study may be of interest also in the field of biochemical taxonomy, i.e. that they may contribute to the distinction of more or less strictly related *Leptodactylus* species or, in other words, may help in the classification of these species. A glance at Figs. 1–4 and Table 2 is sufficient to confirm the correctness of this statement. It is evident that it will be often adequate to have a dry skin at one's disposal to be able to give a precise identification of the *Leptodactylus* species or sub-species to which this skin belongs.

This is only one of the examples supporting the validity of our approach to a biochemical taxonomy of amphibians through screening of the content of their skin in biogenic amines and active polypeptides. Extensive reports on this topic will appear elsewhere.

Note added in press—Quite recently the imidazole spot 1 of the chromatograms of *Leptodactylus pentadactylus labyrinthicus* (cf. Fig. 1) could be identified with N-acetylhistamine, a histamine derivative so far found only in urine.

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