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The Identification of New Histamine Derivatives in the Skin of Leptodactylus[1]

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Extracts of the skin of some South American amphibians belonging to the genus *Leptodactylus* contain, in addition to 5-hydroxyindolealkylamines and hydroxyphenylalkylamines, remarkable amounts of imidazolealkylamines. The species most rich in these compounds are *Leptodactylus pentadactylus labyrinthicus* and *Leptodactylus laticeps*. The skin of the former species contains not only histamine, N'-methylhistamine and N',N'-dimethylhistamine but also two imidazo-*c*-pyridine derivatives hitherto unknown in nature: *spinaceamine* and *6-methylspinaceamine*. The new findings permit a notable enlargement of our knowledge in the field of biogenic imidazolealkylamines and illustrate new possible metabolic pathways for histamine.

During a systematic investigation on biogenic amines and active polypeptides in the amphibian skin it was found that acetone extracts of the skin of *Leptodactylus pentadactylus labyrinthicus*, besides containing small amounts of leptodactyline (1) and considerable amounts of 5-hydroxytryptamine (5-HT), also contained large amounts of imidazole derivatives. Imidazole compounds were also found in *Leptodactylus laticeps* and, to a much lesser extent, in *Leptodactylus pentadactylus dengleri* and *Leptodactylus pentadactylus pentadactylus*.

The present communication describes the techniques which were applied for the identification and the quantitative estimation of five of the imidazole derivatives present in the skin extracts and gives a tentative schematic representation of the biochemical correlations existing among the different compounds. A preliminary report on this topic has already appeared (2).

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MATERIALS AND METHODS

The amphibian material considered in this study was as follows:

(1) *Leptodactylus pentadactylus labyrinthicus:* 7 adult specimens (weight of the dry skins = 73 g.) captured in Misiones, Argentina, in February, 1961 and 5 adult specimens (dry skins = 32 g.) captured at the same place in September, 1961.

(2) *Leptodactylus pentadactylus pentadactylus:* 5 adult male specimens (dry skins = 51.9 g.) captured at Iquitos, Peruvian Amazonia, in September, 1962.

(3) *Leptodactylus pentadactylus dengleri:* 1 adult specimen (dry skin = 5.4 g.) captured in Costa Rica in May, 1962.

(4) *Leptodactylus laticeps:* 1 adult female specimen (dry skin = 5.6 g.) captured in Misiones in February, 1961.

The fresh skins were carefully spread out

and dried in the shade. Immediately after their arrival in Italy by air mail they were minced with scissors and then immersed in 8 parts (w/v) of 70% acetone. The liquid was decanted after a week, and the skins were extracted for another week with 5-6 parts of the solvent. The acetone extracts, brown yellow in color, were combined and filtered. Kept in dark bottles and refrigerated, they may be stored for months without appreciable loss of biogenic amines.

Alkaline alumina was a chromatographic grade product obtained from Merck A.G., Darmstadt. Chromatographic columns were of different size according to the amount of the material to be chromatographed. For amounts exceeding 20 g. of dry skin, the columns were 3.3 cm. wide and 50 cm. high and the alumina weighed 140 g.

The ascending unidimensional technique on Whatman No. 1 paper was routinely employed. Chromatograms were run at 18°C. for 20–30 hours. The following solvents were used: *n*-butanol-30% methylamine (80:30); 1-pentanol-pyridine-water-30% methylamine (40:40:10:1); benzyl alcohol-*n*-butanol-30% methylamine (30:50:30); ethanol - ethyl ether - water - 30% methylamine (40:50:10:0.5); methylethylketone - pyridine - water - 30% methylamine (65:15:10:0.5); isopropanol-0.2 *M* ammonia (30:10); *n*-butanol-pyridine-water (60:30:10); *n*-butanol-acetic acid-water (40:10:50); *n*-butanol-ethanol-acetic acid-water (80:20:10:30).

Paper chromatograms were sprayed with the following developing reagents: (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) 0.05–0.1% alcoholic solution of dichloroquinone chlorimide (Gibbs reagent) followed by sodium carbonate; (d) 1–2% alcoholic solution of *p*-dimethylaminobenzaldehyde, followed by exposure of the chromatograms to HCl vapours in a glass cabinet; (e) 0.2–0.4% solution of Heinrich and Schuler NNCD reagent (2 - chloro - 4 - nitro - 1 - diazobenzene - α - naphthalene sulfuric acid) in 0.1 *M* HCl; (f) diluted Folin-Ciocalteu reagent, followed by sodium carbonate or exposure to ammonia vapours; (g) 1% alcoholic solution of Folin reagent for aminoacids (1,2-naphtoquinone-4-sulfonic sodium salt) followed by sodium carbonate. All the solvents and reagents were of the analytical grade.

Imidazole derivatives are characterized by the positivity of reactions (a), (b), and eventually (g), as well as by the negativity of all other reactions; phenolic derivatives by the positivity of reactions (a), (b), (f), and eventually (c) and (g); 5-hydroxyindole compounds by the positivity of all the tested reactions.

Semiquantitative estimation of imidazoles on paper chromatograms was carried out by visual comparison of the imidazole spots produced by different amounts of crude or purified skin extracts with the spots produced by different known amounts of the corresponding pure synthetic compounds.

Quantitative colorimetric estimation of the imidazole compounds directly in extracts and eluates was performed with the Pauly-Mcpherson's method as modified by Porath (3). Two-tenths ml. of a 1% solution of sulfanilic acid in 10% HCl and 0.2 ml. of a 5% aqueous solution of sodium nitrite are added to 0.1 ml. of a suitably concentrated eluate or extract previously brought to pH 6.5 with *M* HCl. The mixture is shaken for 1-2 minutes and then allowed to stand in the refrigerator for 30 minutes, after which 0.5 ml. of 20% sodium carbonate and 5 ml. of 50% ethanol containing 0.2% sodium carbonate are added. The developing pink or orange red color is read in a Beckman spectrophotometer at 490 and 420 mμ., respectively.

The isolated guinea-pig ileum suspended in 10 ml. of Krebs solution containing 10^{-7} atropine and 10^{-7} 2-bromolysergic acid diethylamide (BOL) was the smooth muscle preparation generally used in the qualitative and quantitative bioassay of imidazolealkylamines.

The following imidazole compounds were synthetized in our laboratory: *N'*-methylhistamine·2 HCl (0.63); *N',N'*-dimethylhistamine·2 HCl (0.66); 4-imidazoleethyltrimethylammonium·Cl·HCl (0.76); 4,5,-6,7-tetrahydroimidazo [5,4-*c*] pyridine. 2

$HCl \cdot H_2O$ (0.57); 6-methyl-4,5,6,7-tetrahydroimidazo [5,4-*c*] pyridine·2 HCl (0.65); and 4,5,6,7-tetrahydroimidazo [5,4-*c*] pyridine-5-carboxylic acid or *spinacine*. In parentheses are the equivalnets in free bases. The name *spinaceamine* has been suggested (see *Discussion*) for 4,5,6,7-tetrahydroimidazo [5,4-*c*] pyridine, and that of *6-methylspinaceamine* for its 6 methyl derivative.

A complete description of the methods of synthesis and characterization of the above compounds is given in another paper (4). As spinacine is concerned see also Ackermann and Skraup (5). Our synthetic spinacine and spinaceamine were indistinguishable from samples of the same compounds kindly supplied by Professor D. Ackermann, Würzburg, Germany.

L-Histidine and histamine dihydrochloride (0.60) were purchased from Hoffmann-La Roche, Basle, Switzerland.

Experimental

Adsorption on Alkaline Alumina

In a typical experiment, 400 ml. of the *Leptodactylus pentadactylus labyrinthicus* September, 1961 extract, corresponding to 30 g. dry skin, was evaporated under reduced pressure and at 45–50°C, to 40–50 ml., and the remaining aqueous liquid was extracted repeatedly with petroleum ether in order to remove fats. The distillation was then continued until the residue was of syrupy consistence. The residue was taken up in a warm water bath, by stirring in 100 ml. of 99% ethanol and the liquid was passed through a column of alkaline alumina. Elution of the column was effected with successive addition of 400 ml. each of 99 and 95% ethanol, 200 ml. each of 90, 80, 70, 60, 50, 40, and 30% ethanol, and 200 ml. of distilled water. Fractions of 200 ml. were collected.

Paper Chromatography–Color Reactions

Aliquots of the above eluates were suitably concentrated under reduced pressure (1 ml. liquid = 2–5 g. dry skin) and then chromatographed on paper. Figures 1 and 2 give a schematic representation of the imidazole spots developed by the Pauly reagent on chromatograms run with the *n*-butanol-acetic acid-water and the *n*-butanol-methylamine mixtures, respectively. The main imidazole spots are indicated by Roman numerals, the minor spots by Arabic numerals.

Table I shows the R_f values, with 5 different solvent systems, of the Pauly-positive spots and Table II the color reactions produced by 3 different reagents. To permit immediate comparison, the Tables also show R_f values and color reactions of a number of synthetic imidazole compounds.

In addition to the solvents listed in Table I, several other solvents were tried with no advantage or with complete failure. Acid solvents, for example, were soon abandoned owing to the slight mobility of the imidazole compounds, and because synthetic hydrochlorides often gave double spots, irregular in their size. Alkaline solvents proved to be far more satisfactory and have been routinely used.

It clearly appears from the tabulated data that substances making up spots I, III, IV, V, VI and VII are indistinguishable both by R_f values and color reactions from 6-methylspinaceamine, *N*,*N*-dimethylhistamine, spinaceamine, *N*-methyl-histamine, histamine, and histidine, respectively.

This is true even for the solvent mixtures not reported in Table I. In no instance was there any disaccordance between the R_f values of a natural imidazole derivative and the corresponding synthetic compound and in superimposition experiments the single natural and synthetic compounds gave always single spots. Of course, both natural and synthetic imidazole compounds did not present any color reaction on spraying with the *p*-dimethylaminobenzaldehyde, the NNCD and the Folin-Ciocalteu reagents.

The identification of the substance making up spot II is in progress. Although this substance has already been prepared by synthesis, his structure has not been fully elucidated till now. 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.

It may be seen from Table I that the

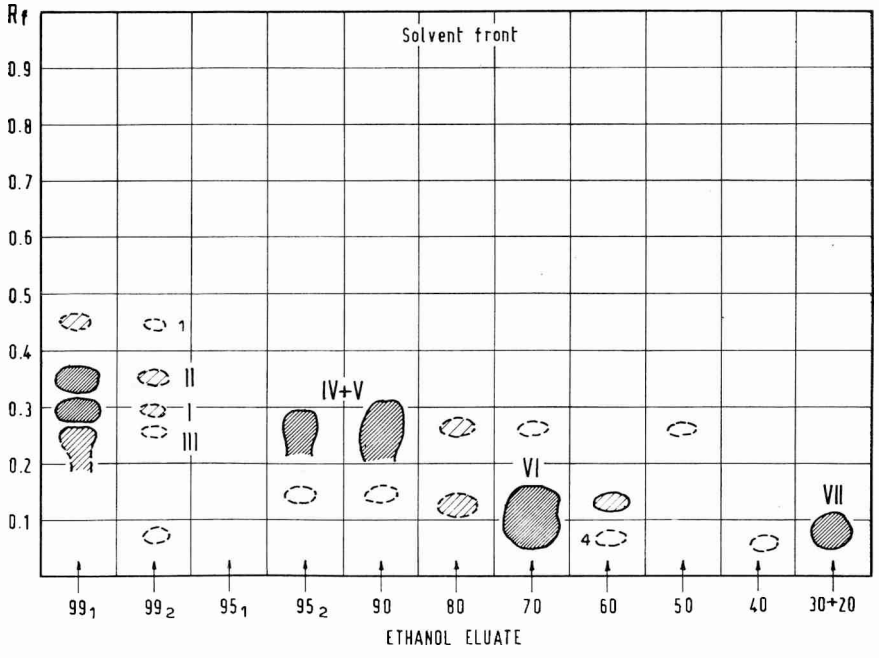


FIG. 1. Paper chromatograms of the ethanol eluates obtained from an alumina column loaded with the skin extract of *Leptodactylus pentadactylus labyrinthicus* Sept., 1961. Solvent, *n*-butanol-acetic acid-water; developing reagent, diazotized sulfanilic acid + sodium carbonate. Amounts of eluates corresponding to 0.1 g. of dry skin were applied on paper at arrows.

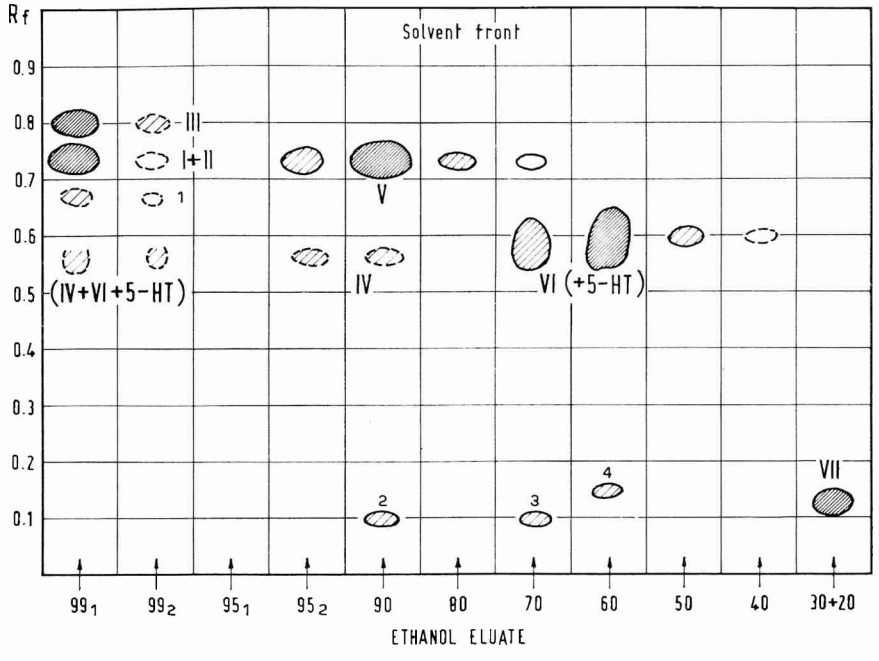


FIG. 2. Paper chromatograms as described in the text of Fig. 1., using as solvent the *n*-butanol-methylamine mixture.

TABLE I

R_f VALUES OF NATURAL AND SYNTHETIC IMIDAZOLE DERIVATIVES WITH DIFFERENT SOLVENTS

	n-Butanol + methylamine	1-Pentanol + pyridine + water + methylamine	Isopropanol + NH_3	Methylethylketone + pyridine + water + methylamine	*n*-Butanol + acetic acid + water
Spot I	0.73–0.78	0.59–0.61	0.75–0.86	0.52–0.55	0.28–0.30
6-Methylspinaceamine	0.78–0.79	0.60–0.64	0.81–0.82	0.49–0.52	double spot
Spot II	0.79–0.80	0.65–0.66	0.75–0.80	0.56–0.62	0.35–0.37
Spot III	0.80	0.68–0.70	0.83–0.86	0.66–0.72	0.24–0.26
N,*N*-Dimethylhistamine	0.82–0.86	0.69–0.74	0.85–0.88	0.66–0.69	double spot
Spot IV	0.54–0.59	0.38	0.63–0.66	0.25–0.26	0.13–0.16
Spinaceamine	0.60–0.62	0.37	0.67–0.69	0.24–0.26	double spot
Spot V	0.72–0.74	0.54–0.55	0.73–0.78	0.49–0.51	0.23–0.25
N-Methylhistamine	0.74–0.76	0.53–0.56	0.78–0.82	0.48–0.51	double spot
Spot VI	0.55–0.58	0.35–0.37	0.60–0.65	0.55–0.60	0.11–0.13
Histamine	0.59–0.61	0.36–0.38	0.63–0.67	0.54–0.57	double spot
Spot VII	0.16	—	0.41	0.15–0.20	0.07–0.10
Histidine	0.15–0.16	—	0.43	0.15–0.20	0.07–0.10
Spot 1	0.64–0.69	0.30	—	—	0.45–0.46
Spot 2	0.09–0.10	—	—	—	0.10
Spot 3	0.11	—	—	—	
Spot 4	0.16	—	—	—	
4-Imidazoleethyltrimethylammonium	0.09	0.04	0.25–0.29	0.03–0.04	double spot
Spinacine	0.19–0.21		0.45	0.12–0.15	0.10–0.12

TABLE II

COLOR REACTIONS OF NATURAL AND SYNTHETIC IMIDAZOLE DERIVATIVES

Numbers in brackets represent the threshold dose (in μg.) necessary for an appreciable reaction on paper.

	Diazotized sulfanilic acid + sodium carbonate	Diazotized *p*-nitroaniline + sodium carbonate	Folin reagent
Spot I	Orange yellow turning into orange red	Light brown	Emerald green
6-Methylspinaceamine	Orange yellow turning into orange red [2]	Light brown [2–3]	Emerald green [5–10]
Spot II	Pink red	Brownish violet	Light blue
Spot III	Pink red	Brownish violet	? (Pale pink)
N,*N*-Dimethylhistamine	Pink red [<1]	Brownish violet [1]	? (Pale pink) [20–30]
Spot IV	Orange yellow turning into orange red	Brownish violet	Rose
Spinaceamine	Orange yellow turning into orange red [2]	Brownish violet [2–3]	Rose [4–6]
Spot V	Pink red	Brownish (rose)	Rose
N-Methylhistamine	Pink red [<1]	Brownish (rose) [<1]	Rose [5]
Spot VI	Pink red	Brownish violet	Gray blue
Histamine	Pink red [<1]	Brownish violet [1]	Gray blue [2]
Spot VII	Pink red	Brownish violet	Brown
Histidine	Pink red [<1]	Brownish violet [1]	Brown [2–3]
Spot 1	Pink red	—	—
Spot 2	Pink red	—	—
Spot 3	Pink red	—	—
Spot 4	Pink red	—	—
4-Imidazoleethyltrimethylammonium	Pink red [1]	Brownish violet [1–2]	? (Pale pink) [50–100]
Spinacine	Orange yellow turning into orange red [2]	—	Brownish [10–15]

imidazole compounds which are more difficult to distinguish by paper chromatography are represented by *N*-methylhistamine and 6-methylspinaceamine, and by histamine and spinaceamine, respectively. However, histamine may be distinctly separated from spinaceamine by the methylethylketone solvent and, on the other hand, a sharp distinction between *N*-methylhistamine and 6-methylspinaceamine, the two nearest compounds in their R_f values in all the tested solvents, is made easy by the Folin reaction, pink for *N*-methylhistamine and emerald green for 6-methylspinaceamine. The same reaction is also useful in distinguishing between the other imidazole compounds. From experiments we have carried out on numerous imidazole-, indole-, and phenyl-alkylamines it seems that primary amines as a rule give bluish or grey-blue colors, secondary amines pink shades, and tertiary amines as well as quaternary ammonium bases only slowly developing faint colors or no color reaction at all.

Histamine, *N*-methylhistamine, and *N*,*N*-dimethylhistamine behave in their adsorption on alumina exactly like the corresponding 5-hydroxyindolealkylamines. In fact, bufotenine is not adsorbed, like *N*,*N*-dimethylhistamine, by alumina and hence appears in the first eluate; 5-HT is eluted, like histamine, by 80–70% ethanol; and *N*-methyl-5-HT is eluted, again like *N*-methylhistamine, by an intermediate ethanol concentration.

Figures 1 and 3 show that alumina column allows a clear-cut separation of four groups of imidazole derivatives: *N*,*N*-dimethylhistamine + 6-methylspinaceamine + compound II; *N*-methylhistamine + spinaceamine; histamine; histidine. Thus, the successive use of alumina chromatography and paper chromatography allows a fully satisfactory distinction among the different imidazole derivatives considered in this study.

Pharmacological Actions

Eluates 99_1, 90, and 70 were separately chromatographed on paper using the *n*-butanol-methylamine mixture as solvent,

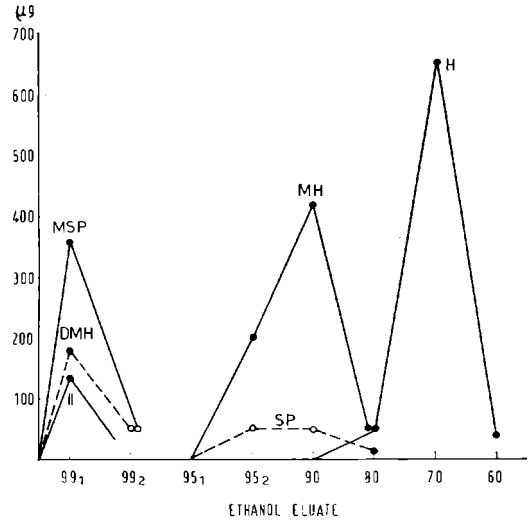


FIG. 3. Recovery of imidazolealkylamines (in μg. per g. dry skin of *Leptodactylus pentadactylus labyrinthicus* Sept., 1961) in the ethanol eluates of an alumina column. 6-Methylspinaceamine (MSP), *N*,*N*-dimethylhistamine (DMH) and compound II (II) were not adsorbed by the column, spinaceamine (SP) and *N*-methylhistamine (MH) were eluted by 95 and 90% ethanol, histamine (H) by 70% ethanol.

and spots I + II, III, IV, V, and VI were separately eluted with Krebs solution. Spot II was also eluted from chromatograms run with *n*-butanol-acetic acid-water. The eluates were assayed on the guinea-pig ileum. It was shown that:

(a) Eluates of spots III, V, and VI displayed a powerful stimulant action which was completely blocked by mepyramine 10^{-7}. For spots giving a Pauly reaction of the same intensity, biological activity was maximum (100%) for spot VI, 60–80% for spot V, and approximately 30% for spot III. This corresponds satisfactorily to the activity ratios existing between histamine and *N*-methylhistamine, and between histamine and *N*,*N*-dimethylhistamine, respectively.

(b) Eluates of spots I, II and IV were virtually inactive, like synthetic spinaceamines[2].

Thus, bioassay offers strong additional

[2] Bertaccini, G., and Vitali, T., unpublished data.

TABLE III

COLUMN CHROMATOGRAPHY OF THE SKIN EXTRACT OF *LETOTADCTYLUS PENTADACTYLUS LABYRINTHICUS* Sept., 1961[a]

Imidazole compound	Imidazolealkylamine content (in μg. free bases per g. dry skin) in ethanol eluates								
	99_1	99_2	95_1	95_2	90	80	70	60	Total
6-Methylspinaceamine									
p.ch.	350–375	50	0[b]	0	0	0	0	0	400–425
sp.m.	330	–	–	–	–	–	–	–	–
Compound II									
p.ch.	125–150	10	0	0	0	0	0	0	135–160
N,*N*-Dimethylhistamine									
p.ch.	170–190	40–50	0	0	0	0	0	0	210–240
sp.m.	150	–	–	–	–	–	–	–	–
b.	160	–	–	–	–	–	–	–	–
Spinaceamine									
p.ch.	10	1–2	0	50	50	10	1	0	120
N-Methylhistamine									
p.ch.	0	0	0	200	420	50	0	0	670
sp.m.	–	–	–	–	370	–	–	–	–
b.	–	–	–	180	350	–	–	–	–
Histamine									
p.ch.	15–20	0	0	0	0	50	650	40	740
sp.m.	–	–	–	–	–	–	550	–	–
b.	–	–	–	–	–	50	560	35	–

[a] The imidazolealkylamine content of the ethanol eluates, as determined by paper-chromatography (p.ch.), Pauly-Porath spectrophotometric method (sp.m.), and bioassay (b.).

[b] 0, not detectable; –, not estimated.

support in favor of the previously suggested identification of the imidazole compounds in the *Leptodactylus* skin.

Quantitative Estimation of the Imidazolealkylamine Content of the Leptodactylus *Skin*

This was carried out chiefly by visual comparison of imidazole spots produced on paper chromatograms by the different ethanol eluates with spots produced by known amounts of the corresponding synthetic imidazole compounds. When possible, semi-quantitative paper chromatographic estimation was checked and completed by spectrophotometric estimation and by bioassay.

Results are shown in Table III and in Fig. 3. It may be seen that values obtained with different methods are in fairly good accordance, although values given by the semiquantitative paper chromatographic method are always somewhat higher than those given by the two other more accurate methods.

The content of imidazolealkylamines in eluates of *Leptodactylus pentadactylus labyrinthicus* February, 1961 and of other strictly related sub-species and species is shown in Table IV.

It is evident that there are striking quantitative and qualitative differences in the imidazolealkylamine content of the different *Leptodactylus pentadactylus* subspecies and that conspicuous quantitative differences may be found even among different batches of *Leptodactylus pentadactylus labyrinthicus*. *Leptodactylus laticeps* appears to be particularly rich in compound II.

TABLE IV

THE CONTENT OF IMIDAZOLEALKYLAMINES[a] IN THE SKIN OF *LEPTODACTYLUS PENTADACTYLUS* AND *LEPTODACTYLUS LATICEPS* AS DETERMINED BY VISUAL COMPARISON OF CHROMATOGRAPHIC SPOTS

	Histamine	*N*-Methyl-histamine	*N*,*N*-Dimethyl-histamine	Spinaceamine	6-Methyl-spinaceamine
Lept. pentad. labyrinthicus					
February, 1961	100	100–120	30–40	20	25–30
September, 1961	740	670	210–240	120	400–425
Lept. pentad. pentadactylus	10–20	n.d.[b]	n.d.	n.d.	n.d.
Lept. pentad. dengleri	35	n.d.	n.d.	n.d.	n.d.
Lept. laticeps	260–280	n.d.	n.d.	5–10(?)	n.d.

[a] In μg. free bases per g. dry tissue.
[b] n.d. = not detectable (<1–2 μg./g.).

TABLE V

THE CONTENT OF INDOLEALKYLAMINES AND PHENYLALKYLAMINES[a] IN THE SKIN OF *LEPTODACTYLUS PENTADACTYLUS* AND *LEPTODACTYLUS LATICEPS*

	Indolealkylamines		Phenylalkylamines	
	5-HT	Bufotenidine[b]	Leptodactyline	Candicine
Lept. pentadactylus labyrinthicus				
February, 1961	200	n.d.[c]	1.3–1.8	n.d.
September, 1961	1800–2000	n.d.	12.5	n.d.
Lept. pentadactylus pentadactylus	130–150	n.d.	9	40–50
Lept. pentadactylus dengleri	50–65	600	11–13	2–3(?)
Lept. laticeps	280	n.d.	2.2–3	n.d.

[a] In μg. free base per g. dry tissue.
[b] Expressed as 5-HT.
[c] n.d. = not detectable (<1–2 μg./g.).

No detectable amounts of imidazolealkylamines could be found in skin extracts of *Leptodactylus ocellatus*, *L. chaquensis*, *L. podicipinus podicipinus*, *L. podicipinus petersi*, *L. rubido cope*, *L. melanonotus*, *L. prognatus* and *L. bufonius*.

Data reported in Table V will help to give a more complete idea about all the biologic amines occurring in the skin of *L. pentadactylus* and *L. laticeps*. They will be discussed in detail in other papers.

DISCUSSION

Results obtained in the present study have substantially enlarged our knowledge in the field of biogenic imidazolealkylamines. In fact, it has been demonstrated that amphibian skin, which is a vertebrate tissue, may contain the following imidazole derivatives: histamine; *N*-methylhistamine; *N*,*N*-dimethylhistamine; 4,5,6,7 - tetrahydroimidazo [5,4-*c*] pyridine; 6-methyl-4,5, 6,7-tetrahydroimidazo [5,4-*c*] pyridine; histidine.

Leaving aside the problem of the actual occurrence of *N*-methylhistamine and *N*,*N*-dimethylhistamine in the sponge *Geodesia gigas* (6) and in human urine (7), it is certain that the two above imidazo-*c*-pyridine derivatives were hitherto completely unknown in nature.

The skin of *Leptodactylus pentadactyus labyrinthicus* offers the most complete sample card of imidazolealkylamines one can imagine and an unique opportunity for the understanding of the biosynthetic correlations existing among the different compounds.

It has been known for a long time and has recently been confirmed in this Labora-

tory that amphibian skin may possess potent *N*-methyltransferase activities (5-HT → *N*-methyl-5-HT → bufotenine → bufotenidine; *p*-tyramine → candicine; *m*-tyramine → leptodactyline).

For this reason, in our attempt to identify the many imidazole spots developed on the *Leptodactylus* chromatograms by the Pauly reagent we immediately directed our attention to the *N*-methylated derivatives of histamine, especially since eluates of some spots proved to be furnished with potent biological activity.

Two of the spots could easily be identified by paper chromatography and bioassay with *N*-methylhistamine and *N*,*N*-dimethylhistamine, respectively. So far, the search for the trimethylammonium derivative of histamine has been unsuccessful.

The discovery by Ackermann (8, 9) of spinacine in the liver of the shark *Acanthias vulgaris* and in the tissues of the crab *Crango vulgaris* suggested the possibility that one or more of our chromatographic spots could be made up of imidazo-*c*-pyridine derivatives, i.e., of *N*-methylated histamines with the lateral chain linked through one *N*-methyl group to the carbon atom 5 of the imidazole nucleus.

We could not find any evidence for the occurrence of spinacine in the *Leptodactylus* extracts, but it soon appeared that two imidazole compounds of these extracts were indistinguishable from 4,5,6,7-tetrahydroimidazo [5,4-*c*] pyridine and 6-methyl-4,5,6,7-tetrahydroimidazo [5,4-*c*] pyridine, respectively. For the new imidazo-*c*-pyridine derivatives we suggest the names of *spinaceamine* and *6-methylspinaceamine*, respectively, to denote their strict relationship with Ackermann's spinacine.

So far, attempts to decarboxylate spinacine by homogenates of mammalian and frog tissues (Hanson, personal communication) and to trace decarboxylation derivatives of spinacine in the urine of rats given the amino acid (De Caro, unpublished observations) have been unsuccessful. This, joined to the apparent lack of spinacine in *Leptodactylus* extracts makes a direct derivation of spinaceamine from spinacine highly questionable.

Thus, the biosynthetic pathway most likely to connect the imidazole derivatives herein described is that represented schematically on opposite page.

Amphibian skin is the only localization of the new histamine compounds discovered so far, but it is highly probable that systematic research will trace them in a number of other vertebrate and invertebrate tissues. To begin with, it would be advisable to check according to the extremely simple methods described in this paper the few natural examples of *N*-methylhistamine and *N*,*N*-dimethylhistamine already described in the literature.

Gaddum (10) has recently suggested that there is reason to believe that methylation of the amino group is the most important mechanism by which histamine is inactivated in the body. The present data offer strong support to this view.

The possible importance of results obtained in this and similar studies as a basis for a biochemical taxonomy of amphibians will be discussed in detail elsewhere.

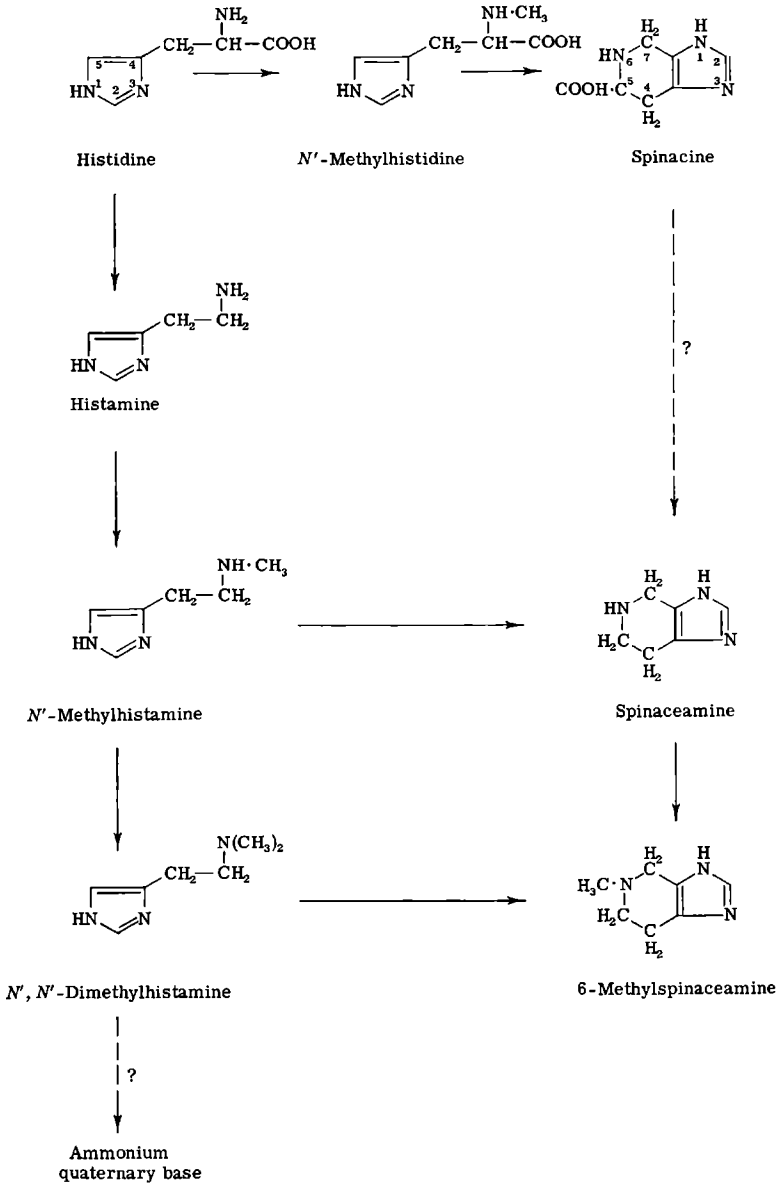
Note added in proof. Since this paper was submitted for publication further progress has been made in the study of the imidazolealkylamines of the *Leptodactylus pentadactylus labyrinthicus* skin:

(1) Spot 1 of the chromatograms could be identified with *N*-acetylhistamine, a histamine metabolite so far found only in urine.

(2) Comparative experiments on the yield of imidazolealkylamines following extraction of a pool of minced dry skins of *Leptodactylus pentadactylus labyrinthicus* (December, 1963) with 70% acetone and 80% methanol, respectively, gave the following results:

	70% acetone	80% methanol
	(μg./g. dry tissue)	
Histamine	50	120
N-Methylhistamine	200	480
N,*N*-Dimethylhistamine	80	140
Spinaceamine	14	6
6-Methylspinaceamine	75	30
5-Hydroxytryptamine	1500	1400

It clearly appears that, whereas extraction of histamine, *N*-methylhistamine, and *N*,*N*-dimethyhistamine is more complete with methanol, that of spinaceamine and 6-methylspinaceamine is more complete with



acetone. Extraction of 5-HT is the same with the two solvents.

REFERENCES

1. ERSPAMER, V., *Arch. Biochem. Biophys.* **82,** 431 (1959).
2. ERSPAMER, V., VITALI, T., ROSEGHINI, M., AND CEI, J. M., *Experientia* **19,** 346 (1963).
3. PORATH, J., *Biochim. Biophys. Acta* **39,** 193 (1960).
4. VITALI, T., AND BERTACCINI, G., *Gazzetta Chim. Ital.*, in press.
5. ACKERMANN, D., AND SKRAUP, S., *Z. Physiol. Chem.* **284,** 129 (1949).
6. ACKERMANN, D., HOLTZ, F., AND REINWEIN, H., *Z. Biol.* **82,** 278 (1924).
7. KAPELLER-ADLER, R., AND IGGO, B., *Biochim. Biophys. Acta* **25,** 394 (1957).
8. ACKERMANN, D., *Z. Physiol. Chem.* **328,** 275 (1962).
9. ACKERMANN, D., AND MÜLLER, E., *Z. Physiol. Chem.* **268,** 277 (1941).
10. GADDUM, J. H., *Proc. Intern. Union Physiol. Sci.* **1,** 849 (1962).