

5-METHOXY- AND 5-HYDROXYINDOLES IN THE SKIN OF *BUFO ALVARIUS**

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Abstract—The skin of *Bufo alvarius*, a desert toad of Arizona, contains a number of indolealkylamines and their metabolites belonging to the common series of 5-hydroxy-indolealkylamines and to the unusual series of 5-methoxyindolealkylamines.

The most abundant representative of 5-hydroxyindolealkylamines is, as in numerous other toads, bufotenine (up to 3 mg per g dry skin), the most abundant representative of 5-methoxyindolealkylamines, *O*-methylbufotenine. In parotoid and coxal glands as much as 5–15 per cent of the dry weight is made up by this compound. Natural *O*-methylbufotenine has been isolated in a pure form and its identity with synthetic *O*-methylbufotenine definitely established.

The *B. alvarius* skin presents three sulphur-containing indolealkylamines: one is bufoviridine, the well known *O*-sulphate of bufotenine, the other two are completely new compounds with sulphuric acid probably attached to the >NH group of the indole nucleus.

All the hitherto described metabolites arising from the oxidative deamination of 5-hydroxy- and 5-methoxy-indolealkylamines may be found in the *B. alvarius* skin: 5-hydroxytryptophol, 5-hydroxyindoleacetic acid, 5-methoxytryptophol and 5-methoxyindoleacetic acid.

The occurrence of the above compounds points to the necessary presence in *B. alvarius* skin of a number of enzymes: tryptophan 5-hydroxylase, catalysing the formation of 5-hydroxytryptophan, aromatic L-aminoacid decarboxylase producing the decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine, *N*-methyl transferase and 5-hydroxyindole-*O*-methyl transferase giving origin to the *N*-methyl and *O*-methylindolealkylamines, and finally sulphaconjugases catalysing the linkage of sulphuric acid to the 5-hydroxy group and the >NH group of the indole nucleus.

The exceptionally rich sample card of indolealkylamines in the skin of *B. alvarius* seems of interest not only from the point of view of comparative biochemistry, but also from that of comparative enzymology and biochemical taxonomy.

Bufo alvarius Girard is a desert toad with a rather restricted area of distribution, covering Arizona (U.S.A.) and Sonora (Mexico).

Among the 40 species of *Bufo* so far examined in this laboratory for the content of their skin in biogenic amines it seems to occupy an unique position, as it is the only species which contains 5-methoxyindolealkylamines.

The occurrence of enormous amounts of these derivatives in the skin of *B. alvarius* is of particular interest because it necessarily involves the concomitant appearance of a considerable 5-hydroxyindole-*O*-methyl transferase activity.

* A preliminary account of part of this work has appeared in *Experientia* 21, 504 (1965).

This paper describes the identification in skin extracts of *B. alvarius* of a number of 5-hydroxy- and 5-methoxyindole derivatives as well as the isolation of *N,N*-dimethyl-5-methoxytryptamine or *O*-methylbufotenine.

It will be seen that the skin of *B. alvarius* is of interest not only because of its content of 5-methoxyindoles but also because it possibly offers the first example of the occurrence in nature of indole derivatives bearing a $-\text{SO}_3\text{H}$ group attached to the nitrogen of the indole nucleus, i.e. in position 1. The presence of these derivatives requires the activity of hitherto unknown conjugases.

MATERIALS AND METHODS

Standard compounds

The following synthetic compounds were available for comparison: tryptamine HCl (0.81), 5-hydroxytryptamine creatine sulphate (5-HT, 0.43), bufotenine base, 5-methoxytryptamine HCl (5-MT, 0.84), *N*-methyl-5-methoxytryptamine HCl (*N*-methyl-5-MT, 0.85), *N,N*-dimethyl-5-methoxytryptamine HCl (*O*-methylbufotenine, 0.86), *N,N*-dimethyl-5-methoxytryptamine methiodide (*O*-methylbufotenidine, 0.69), 5-hydroxyindoleacetic acid (5-HIAA), 5-methoxyindoleacetic acid (5-MIAA), 5-hydroxytryptophol, 5-methoxytryptophol, 5-hydroxytryptophan, tryptophan, histamine 2HCl (0.60), histidine and *N*-acetylhistidine. The amount of free bases in a g of salt is given in parentheses.

The methoxytryptamines were synthesized by one of us (Vitali). Samples of 5-hydroxytryptophol were kindly set at our disposal by Dr. Pletscher (Basle, Switzerland) and Dr. Kveder (Zagreb, Yugoslavia). A sample of 5-methoxytryptophol lightly contaminated by 5-hydroxytryptophol was obtained by treating a few mg of 5-hydroxytryptophol with diazomethane.

N-Methyl-5-hydroxytryptamine (*N*-methyl-5-HT), bufotenidine, bufotenine *O*-sulphate (bufoviridine), bufothionine and dehydrobufotenine were not available as pure compounds. For comparative purposes, in paper chromatography and paper electrophoresis, semipurified extracts (passage through alumina column) of the skin of the following amphibian species were used: *Bufo granulatus fernandezae* (rich in *N*-methyl-5-HT), *Leptodactylus pentadactylus dengleri* (bufotenidine), *Bufo viridis* (bufoviridine), *Bufo marinus* and *Bufo spinulosus* (dehydrobufotenine and bufothionine).

Bufo alvarius skins

Altogether 21 specimens of *Bufo alvarius* were captured in Arizona during 1964–1966. The fresh skins were spread out and dried in the shade.

The single dried skins were, immediately after their arrival in Italy by air mail, minced with scissors and immersed in 20 parts (w/v) of 70% acetone, always keeping distinct the major cutaneous glands (parotoid glands + coxal glands) from the remaining skin. The liquids were decanted after 5–7 days, and the material was re-extracted for another week with 15 parts of the same solvent. A minor part of the 42 extracts was put aside for individual estimations, the bulk was pooled to constitute a glandular and a non-glandular skin extract, respectively.

Chromatography on alumina column

A first partial separation of the numerous indole derivatives occurring in the acetone extracts of *B. alvarius* skin was obtained by their passage through an alkaline

alumina column. 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 10 g of dry non-glandular skin or 3–5 g of cutaneous glands, the columns were 3.3 cm wide and 50 cm high and the alumina weighed 140 g.

Paper chromatography

The ascending unidimensional technique on Whatman No. 1 paper was routinely employed. Chromatograms were run at 18° for 10–30 hr using as a rule the following solvent mixtures: *n.* butanol-acetic acid-water (40:10:50), *n.* butanol-30% methylamine (80:30), 1-pentanol-pyridine-water-30% methylamine (40:40:10:1) and KCl 20% in water. For special purposes also distilled water and methylethylketone-pyridine-water-30% methylamine (65:15:10:0.5) were employed.

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-nitrobenzenediazonium-naphthalene-2-sulphonate); (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*-dimethylaminobenzaldehyde, followed by exposure of the chromatograms to HCl vapours in a glass cabinet; (g) 1% alcoholic solution of Folin reagent for aminoacids (1,2-naphthoquinone-4-sulphonic sodium salt) followed by sodium carbonate; (h) diluted Folin-Ciocalteu reagent, followed by sodium carbonate or exposure to ammonia vapours; (i) 0.2% solution of barium chloride in 70% ethanol followed by 0.05% sodium rhodizonate in 50% ethanol (reagent for sulphates which result in yellow spots on a reddish background); (j) saturated aqueous solution of Reinecke salt, and finally (k) 2% aqueous solution of picric acid. Spraying of reagents (j) and (k) was followed, after some minutes, by gentle washing of the paper sheets under running tap water until complete removal of the background colour.

Thin-layer chromatography

Thin-layer chromatography was carried out on inactive 0.25 mm silica gel (Kieselgel G, Merck) at room temperature. The solvents used were: benzene-ethanol-30% methylamine (22:7:1), *n.* butanol-acetic acid-water (40:10:50), and chloroform-methanol saturated with NH₃ (20:1).

The plates were dried and sprayed either with the NNCD reagent or the *p*-dimethylaminobenzaldehyde reagent.

Gas chromatography

A Fractovap model C gas chromatograph equipped with a Paid/f hydrogen flame ionization detector and with a 1.5 mV Speedomax G recorder was used in all experiments. The columns, 25 cm × 5 mm glass tubes, as well as the support, Chromosorb W 60–80 mesh, were inactivated with dichlorodimethylsilane in toluene followed by an anhydrous methanol rinse. The dry siliconized support was coated by a slurry

filtration process, employing 5% Carbowax 20M in toluene, dried at 110° for 4 hr and packed in the tube which was then equilibrated for 12 hr at 220° under continuous nitrogen flow.

Routinely, chromatographic determinations were carried out under the following conditions: injection port temperature was 265°, column temperature 220°; dry nitrogen was used as a carrier gas at a flow rate of 25 ml/min; hydrogen (0.3 kg/cm²) and air (1 kg/cm²) were provided for the flame ionization detector; samples (1–5 µl.) were injected in diethyl ether or acetone solution.

Light absorption spectra

Ultraviolet absorption spectra were automatically recorded on a Beckman DK-2 spectrophotometer, using 1-cm cells and 10⁻⁴ to 10⁻⁵ solutions in 95% ethanol.

Infrared absorption spectra were run in a Beckman IR-5 spectrophotometer, fitted with rock-salt prism, from pressed discs (KBr) or from solutions (CCl₄) in variable-thickness cell.

X-Ray fluorescence analysis

The X-ray fluorescence analysis (for SK_α) was carried out with a Philips spectrometer, using the Cr radiation with a flow proportional counter and a pentaerythritol analysing crystal.

Paper electrophoresis

A high voltage electrophoresis apparatus from L. Hormuth, Inh. W. E. Vetter (Heidelberg), according to Wieland and Pfeleiderer was used for all the electrophoresis experiments. Paper was the thick Macherey No. 214 paper from Nagel and Co., Düren. Electrophoretic runs were carried out at pH 1.2 (glacial acetic acid–85% formic acid–distilled water 15:25:110), pH 5.8 (99% pyridine–glacial acetic acid–distilled water 15.5:1.8:135), and pH 7.9 (triethylamine–distilled water 5:95; CO₂ was bubbled through until the desired pH was attained).

Hydrolytic procedures

Eluates of alumina columns or of single paper chromatographic spots were occasionally submitted to acid hydrolysis (HCl 0.1–10 M, 10 min–24 hr, at room temperature or 120°; 90–100% acetic acid, 15–120 min at 100°), alkaline hydrolysis (NH₄OH 5 M, 15–60 min at 100°; NaOH 0.05 M, 15 min at 100°) and to treatment with β-glucuronidase (*Helix pomatia* crude enteric juice, bacterial glucuronidase Sigma), 5000–20,000 units, 8–16 hr at 38°.

RESULTS

Adsorption on alkaline alumina

In a typical experiment, 500 ml of the methanol extract of non-glandular skin, corresponding to 15 g of dry tissue, was evaporated, under reduced pressure and at 45–50°, to 30–40 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 200–400 ml each of 99, 95, 90, 80, 70, 60, 50, 40% ethanol, and 200 ml of distilled water. Fractions of 200 ml were collected.

Elution of alumina columns loaded with the extract of non-glandular skin was always easy and the result was a good separation and partial purification of the different indole compounds. Elution of alumina columns loaded with the extract of cutaneous glands, on the contrary, was more difficult owing to obstruction of the column produced, at certain ethanol concentrations, by swelling of a mucilaginous material present in the extract. Moreover, results were unsatisfactory, since minor indole compounds were masked and nearly overwhelmed by the enormous amounts of *O*-methylbufotenine occurring in the glandular tissue.

Paper chromatography and colour 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. Fig. 1 gives a

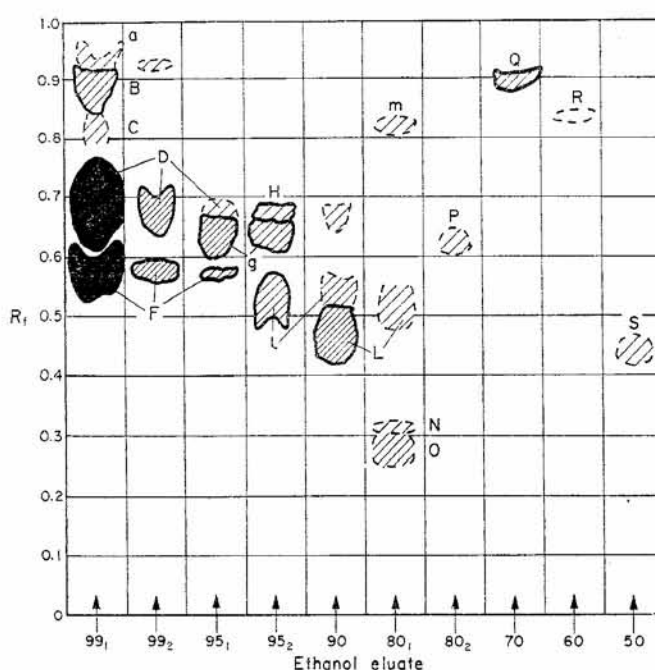


FIG. 1. Paper chromatograms of the ethanol eluates obtained from an alumina column loaded with the extract of pooled non-glandular skin of *Bufo alvarius*. Solvent, *n*. butanol–acetic acid–water; developing reagent, NNCD reagent. Amounts of eluates corresponding to 0.15 g of dry skin were applied on paper at arrows.

schematic representation of the 17 indole spots developed by the NNCD reagent on chromatograms run with the *n*-butanol–acetic acid–water mixture.

Already identified spots are indicated by capital letters, unknown spots by small letters.

Table 1 shows the R_f values, with 4 different solvent systems, of the NNCD-positive spots and Table 2 the colour reactions produced by 5 different developing reagents. To permit immediate comparison, Table 1 also shows the R_f values of eleven pure indole compounds.

TABLE 1. PAPER CHROMATOGRAPHY. R_f VALUES OF THE INDOLE DERIVATIVES OCCURRING IN SKIN EXTRACTS OF *Bufo alvarius* AND OF PURE NATURAL OR SYNTHETIC INDOLE COMPOUNDS

	<i>n.</i> Butanol- acetic acid- water (40:10:50)	1. Pentanol- pyridine-30% methylamine water (40:40:1:10)	<i>n.</i> Butanol- 30% methyl- amine (80:30)	20% Potassium chloride
Spot a	0.92-0.94	—	—	—
Spot B	0.87-0.89	0.90-0.92	0.90-0.92	0.32-0.34
5-Methoxytryptophol	0.87-0.92	0.89-0.93	0.88-0.93	0.35-0.37
Spot C	0.78-0.82	0.84-0.85	0.70-0.72	0.36-0.38
5-Hydroxytryptophol	0.77-0.82	0.86-0.87	0.68-0.74	0.38-0.42
Spot D	0.66-0.68	0.82-0.86	0.89-0.91	0.46-0.48
<i>O</i> -Methylbufotenine	0.67-0.69	0.82-0.88	0.92-0.93	0.46-0.47
Spot e	—	0.47-0.53	0.52-0.54	—
Spot F	0.53-0.57	0.73-0.75	0.83-0.87	0.71-0.74
Bufotenine	0.51-0.56	0.72-0.74	0.81-0.88	0.72-0.73
Spot g	0.62-0.64	0.44-0.52	0.85-0.87	0.74-0.80
Spot H	0.64-0.66	0.65-0.68	0.91-0.93	0.37-0.40
<i>N</i> -Methyl-5-methoxytryptamine	0.62-0.65	0.63-0.66	0.92-0.94	0.39-0.42
Spot I	0.51-0.54	0.56-0.60	0.75-0.81	0.36-0.38
<i>N</i> -Methyl-5-hydroxytryptamine	0.46-0.50	0.55-0.62	0.77-0.82	0.37-0.40
Spot L	0.51-0.54	0.56-0.60	0.75-0.81	0.36-0.38
Spot m	0.79-0.82	0.73-0.75	—	—
Spot N	0.31-0.35	0.51-0.55	0.50-0.52	0.54-0.57
Bufoviridine	0.30-0.32	0.48-0.49	0.47-0.48	0.58-0.62
Spot O	0.27-0.31	0.41-0.46	—	0.61-0.65
Spot P	0.48-0.52	—	0.62-0.64	0.36-0.38
5-Hydroxytryptamine	0.43-0.49	0.56-0.59	0.59-0.66	0.34-0.38
Spot Q	0.89-0.90	—	0.34-0.36	0.50-0.52
5-Methoxyindoleacetic acid	0.87-0.90	0.36-0.45	0.36-0.45	0.50-0.52
Spot R	0.78-0.84	—	0.15-0.17	—
5-Hydroxyindoleacetic acid	0.78-0.80	0.38-0.40	0.15-0.23	0.52-0.54
Spot S	0.45	0.34	0.32	0.55
Tryptophan	0.45	0.37	0.33	0.57

It should be added, completing the data of Table 2, that identical colour reactions were given, respectively, by spot B and 5-methoxytryptophol, spot C and 5-hydroxytryptophol, spot D and *O*-methylbufotenine, spot F and bufotenine, spot H and *N*-methyl-5-methoxytryptamine, spot I and *N*-methyl-5-HT, spot N and bufoviridine, spot P and 5-HT, spot Q and 5-MIAA, spot R and 5-HIAA, and finally spot S and tryptophan.

Hence, substances making up the above eleven spots were chromatographically indistinguishable from the corresponding pure compounds.

Chromatograms loaded with amounts of eluates corresponding to 0.3-0.5 g of dry non-glandular skin were also sprayed with aqueous solutions of picric acid and Reinecke salt, respectively, and then washed gently but thoroughly under running tap water. Upon spraying with solution of picric acid three yellow spots made their appearance, corresponding to spots D, F and L. However, only the picrate given by

spot D proved to be sufficiently water-insoluble to withstand thorough washing with water. Solution of Reinecke salt produced the appearance of five pinkish violet spots, three of them corresponding to indole spots D, F and g, and the other two to unknown non-indolic compounds. All these spots persisted, unchanged, after thorough washing with water. They could be eluted by ethanol or acetone.

TABLE 2. COLOUR REACTIONS OF THE INDOLE DERIVATIVES OCCURRING IN SKIN EXTRACTS OF *Bufo alvarius*

	NNCD reagent	Echtrotsalz B reagent	Pauly reagent	Gibbs reagent	p-Dimethyl-amino-benzaldehyde reagent
Spota	yellowish	light brown yellow	O	O	violet
B	gold yellow	yellow (rose)	light olive yellow	O	blue
C	rose yellow	rose	rose	violet blue	blue
D	orange brown	light brown yellow	light olive yellow	O	blue
e	yellowish	—	—	—	blue
F	peach red	pinkish violet	wine red	violet blue	blue
g	dirty yellow	light brown yellow	O	O	gold yellow
H	orange brown	light brown yellow	light olive yellow	O	blue
I	peach red	pinkish violet	wine red	violet blue	blue
L	slowly developing peach red	O	O	O	blue
m	yellow	yellowish brown	O	O	gold yellow
N	rose	pinkish violet	brick red	brownish violet	blue
O	orange yellow	O	O	O	violet
P	peach red	pinkish violet	wine red	violet blue	blue
Q	orange brown	O	O	O	blue
R	peach red	pinkish violet	wine red	violet blue	blue
S	yellow	O	O	O	violet

O = negative reaction

Spots produced by synthetic *O*-methylbufotenine and bufotenine behaved, towards picric acid and Reinecke salt, exactly like spots D and F, respectively.

Thin-layer chromatography

As shown in Table 3, thin-layer chromatography confirmed the identity of substances constituting spots B, D and H with 5-methoxytryptophol, *O*-methylbufotenine and bufotenine, respectively.

The material put on silica gel plates was obtained by elution of paper chromatographic spots, except for spot D for which the ethanol eluate 99₁ was directly used.

Paper electrophoresis

High voltage paper electrophoresis was carried out either on eluates of alumina columns or on eluates of single paper chromatographic spots. The latter procedure was always necessary for spots B and C.

The electrophoretic run of 5-HT was considered = 1, that of the other compounds was referred to 5-HT. Results obtained at three different pHs (1.2, 5.8 and 7.9) are shown in Table 4.

The tabulated data once again point to the identity of substances constituting spots B, C, D, F and N with 5-methoxytryptophol, 5-hydroxytryptophol, *O*-methylbufotenine, bufotenine and bufoviridine, respectively. They further show that spot g

has a clear basic nature, whilst spots L and O possess a poor electrical motility at every pH.

Spots Q and R migrated towards the anode and in their electrical motility they were indistinguishable from 5-methoxyindoleacetic acid and 5-HIAA, respectively.

TABLE 3. THIN-LAYER CHROMATOGRAPHY. R_f VALUES OF SOME OF THE INDOLE DERIVATIVES OCCURRING IN SKIN EXTRACTS OF *Bufo alvarius* AND OF SYNTHETIC INDOLE COMPOUNDS

	<i>n</i> . Butanol- acetic acid- water (40:10:50)	<i>n</i> . Butanol- 30% methyl- amine (80:30)	Chloroform- methanol saturated with NH ₃ (20:1)	Benzene- ethanol-30% methylamine (22:7:1)
5-Hydroxytryptamine	0.59-0.63	0.75-0.78	—	0.43-0.47
5-Methoxytryptamine	0.59-0.62	0.87-0.90	0.16	0.67-0.68
Spot B	0.95	0.90	0.63	0.70-0.73
5-Methoxytryptophol	0.92-0.93	0.89	0.60	0.69-0.71
5-Hydroxytryptophol	0.95-0.97	0.78-0.85	—	0.05-0.06
Spot D	0.42	0.95	0.50-0.52	0.81
O-Methylbufotenine	0.40	0.97	0.48-0.50	0.79-0.80
Spot F	0.35	0.92	0.15	0.70
Bufotenine	0.40	0.91	0.17	0.66
Spot g	0.60	—	—	0.77
Spot L	0.32	0.65	0.00	—
Spots N/O	0.21-0.25	0.55	0.00	0.09
Bufoviridine	0.30-0.35	0.47-0.52	—	0.05-0.06
5-Hydroxyindoleacetic acid	0.97-0.98	0.32-0.40	—	0.03-0.04
5-Methoxyindoleacetic acid	0.96-0.97	0.42-0.50	0.00	0.07-0.08
Melatonin	0.93	0.94-0.97	0.64	0.68-0.70
Dehydrobufotenine	0.47-0.52	0.59-0.63	—	—

TABLE 4. HIGH VOLTAGE ELECTROPHORESIS. RELATIVE MIGRATION RATES TOWARDS THE CATHODE OF SOME INDOLE DERIVATIVES OCCURRING IN THE SKIN OF *Bufo alvarius* AND OF SOME PURE INDOLE COMPOUNDS (Migration rate of 5-HT = 1)

	pH 1.2	Electrophoretic run at pH 5.8	pH 7.9
5-Hydroxytryptamine	1	1	1
Spot B	0.08-0.09	0.26	0.50-0.55
5-Methoxytryptophol	0.08-0.1	0.27	0.55
Spot C	0.06-0.08	0.26	0.50-0.55
5-Hydroxytryptophol	0.07-0.09	0.26	0.55
Spot D	1	1.08	1.5-1.7
O-Methylbufotenine	1.05	1.1	1.50-1.65
Spot F	0.98	1	1.6-1.7
Bufotenine	0.98-1	1	1.6-1.7
Spot g	1.08-1.1	1.2	2.4-2.5
Spot L	0.01-0.03	0.21	0.3-0.35
Spot N	0.08-0.10	0.24	0.1
Bufoviridine	0.06-0.16	0.12-0.16	0.1-0.2
Spot O	0.12-0.14	0.32	0.25
Dehydrobufotenine	0.96-1	0.86-0.9	0.77-0.84
Bufothionine	—	0.13-0.18	0.1

Semi-quantitative estimation of the indole compounds of the B. alvarius skin

This was carried out chiefly by visual comparison of the individual indole spots produced on paper chromatograms by different amounts of crude skin extracts or

ethanol eluates with the spots produced by known amounts of the corresponding pure synthetic compounds.

Table 5 shows the content of bufotenine and *O*-methylbufotenine in the single examined *B. alvarius* skins (glandular and non-glandular skin).

TABLE 5. THE CONTENT OF BUFOTENINE AND *O*-METHYLBUFOTENINE IN THE SKIN OF THE INDIVIDUAL EXAMINED SPECIMENS OF *Bufo alvarius*

(G = large cutaneous glands; RS = remaining skin)

	Bufotenine (mg base/g dry tissue)		<i>O</i> -Methylbufotenine (mg base/g dry tissue)	
	G	RS	G	RS
I	2.2	1.5	70	2.7
II	1.1	2.1	140	2.6
III	0.9	0.3	85	1.0
IV	2.5	1.0	65	2.3
V	5.0	1.3	90	1.7
VI	3.5	1.4	105	2.0
VII	0.8	1.1	50	2.3
VIII	3.2	0.8	85	1.7
IX	1.4	1.0	105	3.0
X	0.9	0.6	140	1.5
XII	1.4	0.2	50	1.0
XIII	2.0	2.2	70	0.9
XIV	0.4	0.5	130	4.0
XV	0.35	0.75	105	2.0
XVI	0.3	0.55	150	2.2
XVII	0.5	1.2	130	3.5
XVIII	<0.1	0.17	50	0.6
XIX	<0.2	0.62	125	0.42
XX	<0.2	0.50	120	0.75
XXI	<0.2	0.87	90	0.5

The content of other indolealkylamines in the pooled non-glandular skins was as follows, in μg per g of dry tissue: 5-HT base 4–6, *N*-methyl-5-HT base 30–40, *N*-methyl-MT base 10–15, bufoviridine 15–20, methoxytryptophol 100–120, 5-hydroxytryptophol 4–8, 5-HIAA 10–12, and 5-MIAA 40. Orientative estimates of substances making up unknown or not fully known spots gave the following values: spot g 200–300 $\mu\text{g/g}$ (expressed as 5-methoxytryptophol), spot L 60–65 $\mu\text{g/g}$ (expressed, after hydrolysis with acetic acid, as 5-MT), and spot O 50–55 $\mu\text{g/g}$ (expressed as bufotenine, after hydrolysis with acetic acid).

All or nearly all the minor indole compounds occurring in non-glandular skin were also present in extracts of the large cutaneous glands, probably in comparable amounts. However, their semiquantitative estimation was very difficult owing to the heavy predominance of *O*-methylbufotenine.

Isolation and characteristics of O-methylbufotenine

Upon passage through an alumina column of the crude extracts of *B. alvarius* skin, *O*-methylbufotenine emerged, as already stated and shown in Fig. 1, in the first ethanol eluates, i.e. in the 99 and 95% ethanol eluates.

Suitable amounts of these eluates were combined, evaporated to dryness and the residue dissolved in 20 ml of boiling water. Another 20 ml of boiling water containing 0.3 g of picric acid was gradually added. The liquid was rapidly filtered on a

warmed funnel. On cooling, yellow-orange needles made their appearance. After drying they weighed 235 mg. Upon slow recrystallization from boiling water brownish-red needles were obtained, melting at 175–176° (decomposition). Upon rapid recrystallization, needles were yellow-orange in colour, with slightly lower m.p. The two picrates were indistinguishable by thin-layer chromatography.

Molecular weight was calculated according to Cunningham *et al.*,¹ assuming that the brownish-red picrate was a monopicate. The found molecular weight (448.5) was in excellent accordance with the calculated molecular weight of a *N,N*-dimethylmethoxytryptamine (447.4), in particular with that of *O*-methylbufotenine, which gives a monopicate melting at 176.5–177°. ^{2, 3, 4} Per cent N for the picrate of the natural compound: 15.40; calculated for $C_{19}H_{21}O_8N_5$: 15.65.

Forty mg of the natural monopicate were dissolved in 50% ethanol and then passed through a column of a strongly basic ion exchange resin (Merck III resin in the —OH form; 30 g resin in a column of 1.6 cm diameter; height of the resin column 30 cm). Elution was performed with 40% ethanol. The eluates giving a *p*-dimethylaminobenzaldehyde reaction were combined and then concentrated to a small volume. The crystalline precipitate was recrystallized from hexane, giving small colourless prisms, melting at 67.5–68°, in agreement with the melting point of *N,N*-dimethyl-5-methoxytryptamine.^{2, 3, 5}

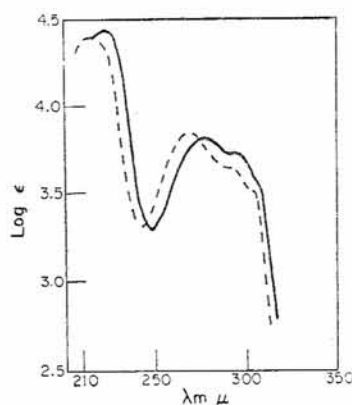


FIG. 2. Ultraviolet absorption spectra of 5-methoxyindole (---) and of natural *O*-methylbufotenine (—). The two compounds present the same absorption maxima.

The ultraviolet absorption spectrum (Fig. 2) of the natural base looked like that of an unmodified 5-methoxyindole,⁶ and the indole >NH stretching band (Fig. 3) was quite evident in the infrared spectrum, from CCl_4 solution.⁷ Moreover, the natural indole gave a single symmetrical peak by gas chromatography, at 138 sec after the injection of the sample in ethereal solution. The inlet of the sample with benzaldehyde or in acetone solution did not produce either shift or change in intensity of peak. This suggests a probable absence of any primary amine function.^{8, 9}

Accordingly, the *N,N*-dimethyl-5-methoxytryptamine was synthesized^{6, 10} and compared with the natural indole. Undepressed melting points for these bases as well as for the corresponding monopicates were observed; and ultraviolet and infrared absorption spectra were the same (Fig. 3). Moreover, the natural base and the

synthetic *O*-methylbufotenine were indistinguishable by either paper chromatography and thin-layer chromatography or gas chromatography.

A last proof of the identity of the indole compound making up spot D with *O*-methylbufotenine was given by its possible transformation into an *N*-oxide indistinguishable from that given by synthetic *O*-methylbufotenine. *N*-Oxidation was carried out by the method of Fish *et al.*,¹¹ with hydrogen peroxide (1 ml 40% H_2O_2 in 10 ml of 95% ethanol).

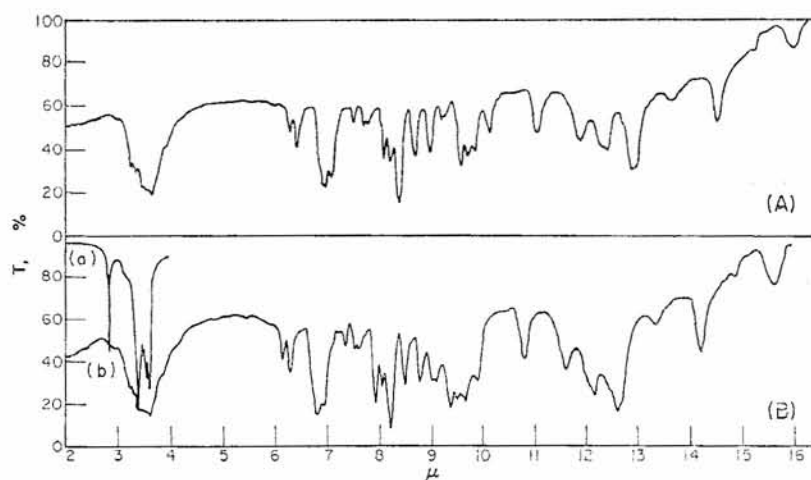


FIG. 3. A. Infrared spectra of synthetic *O*-methylbufotenine from KBr pressed disk. B. Infrared spectra of natural *O*-methylbufotenine: (a) from CCl_4 0.5% solution, 2 mm cell; (b) from KBr pressed disk. The indole $>\text{NH}$ stretching band is quite evident around 2.85μ , from CCl_4 solution.

Tentative identification of substances making up spots L and O

In the assumption that substances constituting spots g, L and O might be derivatives of known indole compounds, paper chromatographic eluates of these spots were subjected to acid hydrolysis, alkaline hydrolysis, and treatment with β -glucuronidase. Preliminary results were as follows:

(a) Treatment with β -glucuronidase did not cause any alteration of eluates of spots g, L and O.

(b) Hydrolysis with hydrochloric acid produced, as expected, a more or less complete destruction of all examined indole compounds with no clearly recognizable hydrolysis products.

(c) Hydrolysis with either ammonia or diluted NaOH solution caused no alteration of substances constituting spots g, L and O.

(d) Hydrolysis with 90–100% acetic acid (15–60 min at 100°) had no effect on spot g, while provoking a quantitative splitting of substances constituting spots L and O. Substance L yielded *O*-methylbufotenine and sulphuric acid, substance O bufotenine and again sulphuric acid.

The occurrence of sulphuric acid in substances L and O was established by the positivity of the rhodizonate reaction and by the results of X-ray fluorescence analysis. In fact, X-ray spectrometric analysis (carried out by Dr. Manfredotti, Institute for Structural Chemistry, University of Parma) showed unequivocally not only the

presence of considerable amounts of sulphur in both L and O chromatographic spots, but also the comparatively higher sulphur content of spot O.

On the other hand, hydrolysates of substances constituting spots L and O did not contain either aminoacids or sugars (pentoses and hexoses), and treatment with acetic acid while producing a quantitative hydrolysis of *O*-sulphates of indoles (bufoviridine and bufothionine), was virtually ineffective on *O*-phosphates (psilocybin), *O*-glucuronides (*O*-glucuronides of 5-HT and 5-HTP) and *O*-acetates (5-acetoxytryptophan), as well as on *N*-acetyl derivatives, such as *N*-acetyltryptophan and melatonin.

It may be tentatively suggested that substance L is *O*-methylbufotenine with a molecule of sulphuric acid attached to the >NH of the indole ring, and substance O bufotenine bearing two molecules of sulphuric acid attached to the indole nucleus, one to the phenolic —OH group, the other to the >NH group.

So far we know, it is the first time that similar compounds are found in nature.

We are presently engaged in a thorough study of the above sulphur-containing indolealkylamines and of related compounds as well. It is obvious that a decisive confirmation of the suggested structures will come from the synthesis. This is in progress. A full report of this part of our studies will be published later on.

DISCUSSION

The skin of *Bufo alvarius* presents an exceptionally rich sample card of indolealkylamines, both from a quantitative and a qualitative point of view.

Two nearly complete series of indolealkylamines occur in the *B. alvarius* skin. The first one, that of the classical 5-hydroxyindolealkylamines, is common not only to a number of toads but also to amphibians belonging to several other genera and families; the second, that of the methoxyindolealkylamines, is so far peculiar for *B. alvarius*.

An unexpected completion of the two above series of indolealkylamines is represented by the appearance of compounds probably carrying sulphuric acid attached to the >NH group of the nucleus.

The biosynthetic pathways which are most likely to connect the indole derivatives herein described are represented schematically below (Figs. 4 and 5).

5-HT, which must be considered the parent substance of all the indolealkylamines of the *B. alvarius* skin, is present only in traces, and similarly very low are the amounts of *N*-methyl-5-HT and *N*-methyl-5-MT, in comparison with those of the corresponding tertiary amines. 5-MT is either lacking or present in traces.

This means that 5-HT once formed is immediately submitted to the combined action of *N*-methyl transferase and of 5-hydroxy-*O*-methyl transferase, the final result being the formation and accumulation of enormous amounts of *O*-methylbufotenine. *N*-Methyltransferase activity does not seem to cause, in neither series of compounds, the formation of quaternary ammonium bases.

Concerning the origin of the *O*-methylated derivatives, both the horizontal and the vertical pathways represented in Fig. 4 are possible. However, according to Axelrod and Weissbach,¹² bufotenine is a better substrate than either 5-HT and *N*-methyl-5-HT for the 5-hydroxyindole-*O*-methyl transferase of the mammalian pineal tissue.

So far, 5-methoxyindoles have been described only in some South American vegetables^{3, 4, 9, 13, 14} and, in animals, only in the pineal gland of mammals and birds.¹⁵⁻¹⁷

According to Axelrod and Weissbach,¹² 5-hydroxyindole-*O*-methyl transferase, the enzyme responsible for the *O*-methylation of 5-hydroxyindoles, is strictly localized

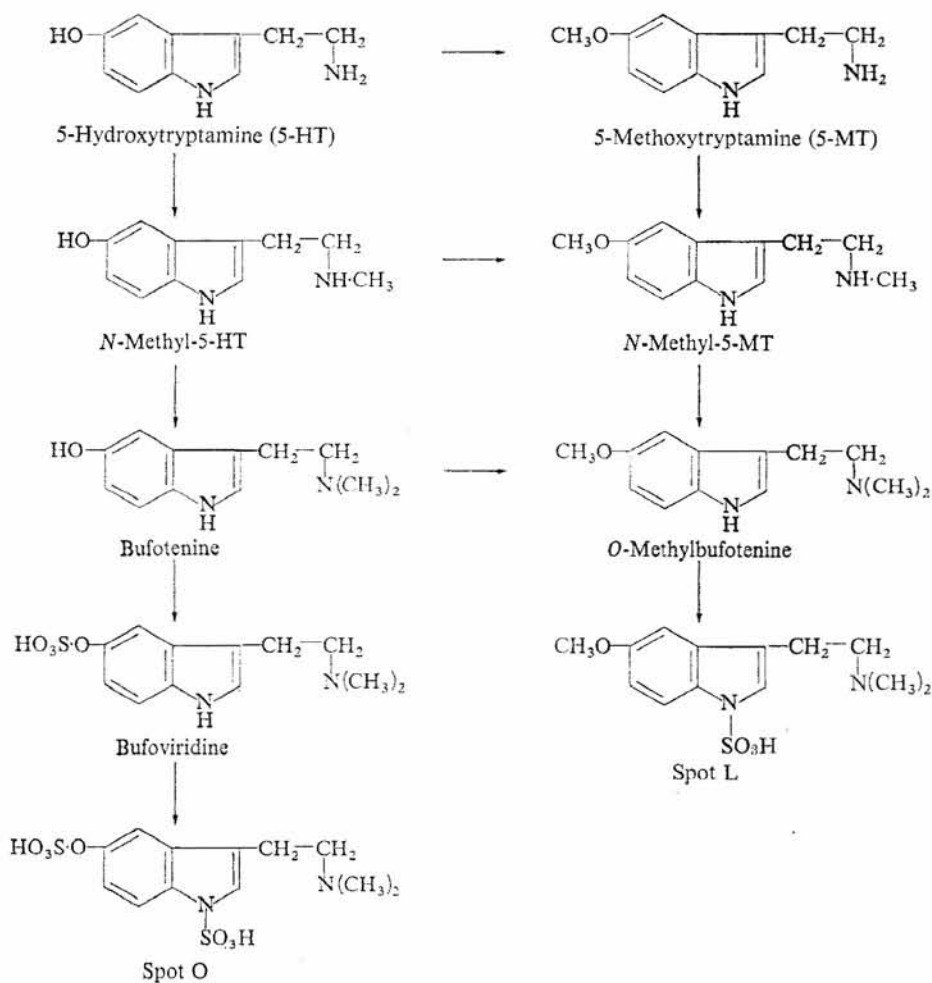


FIG. 4. 5-Hydroxy- and 5-methoxyindolealkylamines of the *Bufo alvarius* skin, and their metabolic pathways.

in the pineal body. Present results, however, show that also amphibian skin may possess intense 5-hydroxyindole-*O*-methyl transferase activity. Although keeping in mind that the turnover rate of the two 5-methoxyindoles may be strikingly different, one cannot avoid to compare the 100-000-150-000 μg of *O*-methylbufotenine contained in the skin of a medium-sized specimen of *B. alvarius* with the probable nanogram amounts of 5-methoxy-*N*-acetyltryptamine (melatonin) present in the pineal body of the same animal.

Thus, it would seem that fresh skin of *B. alvarius* is a suitable material for studying 5-hydroxyindole-*O*-methyl transferase activity and, consequently, the rate of biosynthesis of *O*-methylbufotenine. Enzymic studies *in vitro* should obviously be completed by the search of labelled 5-methoxyindoles following administration to the intact toad of radioactive L-tryptophan or 5-hydroxytryptophan. The negative results

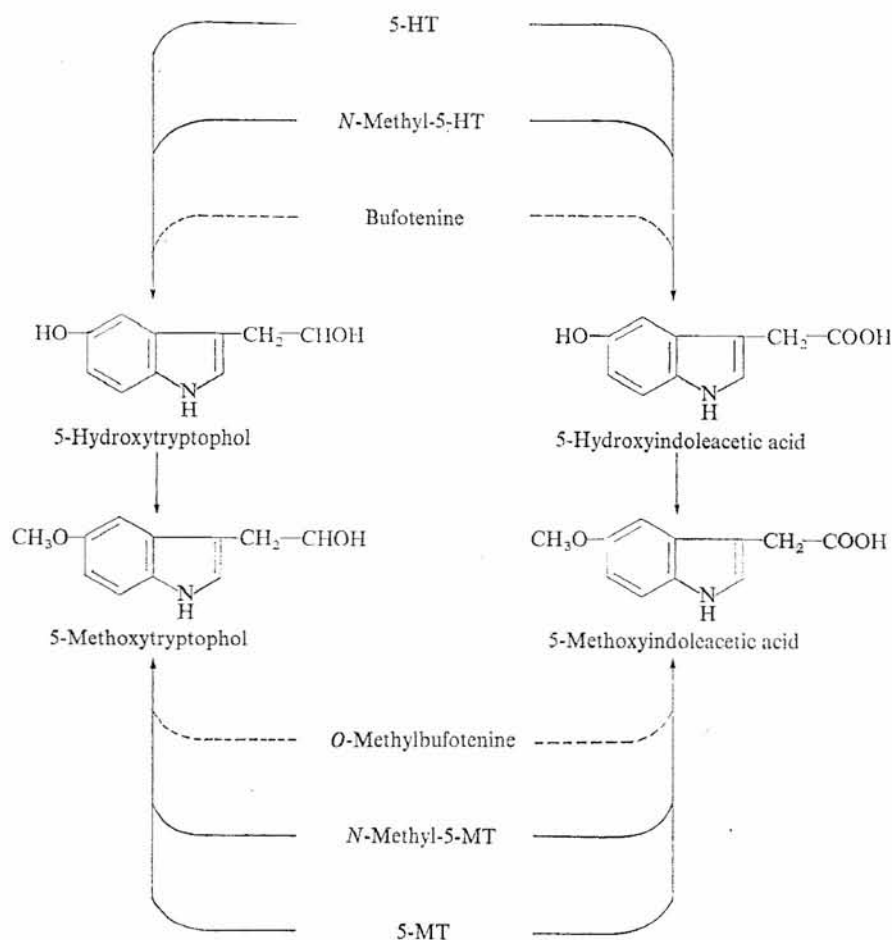


FIG. 5. Metabolites of 5-hydroxy- and 5-methoxyindolealkylamines in the *Bufo alvarius* skin arising from oxidative deamination.

of Märki *et al.*,¹⁸ who failed to detect any 5-hydroxyindole-*O*-methyl transferase activity in the parotoid gland of *Bufo marinus* are not surprising, because no trace of 5-methoxyindole derivatives is present in this species.

The skin of *B. alvarius* contains, in addition to the expected metabolites 5-hydroxyindoleacetic acid and 5-methoxyindoleacetic acid, also trace amounts of 5-hydroxytryptophol and important amounts of 5-methoxytryptophol, the latter compound, so far found only in pineal tissue,¹⁷ being apparently the major metabolite originating from the oxidative deamination of the 5-methoxyindolealkylamines.

The findings of Kveder *et al.*¹⁹ and of McIsaac *et al.*¹⁷ on the occurrence of 5-hydroxytryptophol in rat urine and in pineal tissue as a metabolite of 5-HT, have been confirmed in amphibians.

Among the indole compounds occurring in acetone extracts of the *B. alvarius* skin three remain to be thoroughly investigated. They make up, on paper chromatograms, spots g, L and O.

A number of analytical data, which will be discussed in detail elsewhere, demonstrate that substance making up spot L is a derivative of *O*-methylbufotenine, and substance making up spot O a derivative of bufotenine. Substances L and O are easily hydrolyzed by acetic acid with liberation of sulphuric acid, and all available evidence suggests that this sulphuric acid may be attached either to the phenolic —OH group or to the pyrrolic >NH group or to both. It is obvious that synthetic work will be necessary to confirm the analytical results.

It should be added that spot O is not peculiar for chromatograms of extracts of *B. alvarius* skin. It has been traced, in fact, with an intensity several times greater than that seen on chromatograms of *B. alvarius* skin, also on paper chromatograms of skin extracts of *Bufo marmoratus* and *Bufo perplexus*.

The compound making up spot g is puzzling, among other things owing to its particular colour shade with the *p*-dimethylaminobenzaldehyde reagent. Its behaviour in paper electrophoresis and with Reinecke salt points to its decidedly basic nature. The isolation of this compound, starting from its Reinecke salt, is only a matter of availability of sufficient *B. alvarius* material.

Concerning some minor chromatographic spots, such as spots a, e₁, e₂ and m, the possibility cannot be ruled out that substances making up them do not pre-exist in the living skin, but are artifacts originating during drying or during acetone extraction of the skin.

Research reported in this paper will probably be of some interest also from the point of view of biochemical taxonomy.

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REFERENCES

1. K. G. CUNNINGHAM, W. DAWSON and F. S. SPRING, *J. chem. Soc.* 2305 (1961).
2. T. HOSHINO and K. SHIMODAIRA, *Bull. chem. Soc. Japan* **11**, 221 (1936).
3. I. J. PACHTER, D. E. ZACHARIAS and O. RIBEIRO, *J. org. Chem.* **24**, 1285 (1959).
4. G. LEGLER and R. TSCHESCHE, *Naturwissenschaften* **50**, 94 (1963).
5. F. BENINGTON, R. D. MORIN and L. C. CLARK, *J. org. Chem.* **23**, 1977 (1958).
6. T. VITALI and F. MOSSINI, *Gazz. chim. ital.* **88**, 574 (1958).
7. H. DUNKEN and H. FITSCHE, *Z. Chem.* **2**, 379 (1962).
8. H. M. FALES and J. J. PISANO, *Analyt. Biochem.* **3**, 337 (1962).
9. B. HOLMSTEDT, W. J. A. VANDENHEUVEL, W. L. GARDINER and E. C. HORNING, *Analyt. Biochem.* **8**, 151 (1964).
10. T. VITALI and F. MOSSINI, *Boll. sci. Fac. Chim. ind. Univ. Bologna* **17**, 84 (1959).
11. M. S. FISH, M. M. JOHNSON and E. C. HORNING, *J. Am. chem. Soc.* **77**, 5892 (1955).
12. J. AXELROD and H. WEISSBACH, *Science* **131**, 1312 (1960); *J. biol. Chem.* **236**, 211 (1961).
13. S. WILKINSON, *J. chem. Soc.* 2079 (1958).
14. B. HOLMSTEDT, *Archs int. Pharmacodyn. Théor.* **156**, 285 (1965).

15. A. B. LERNER and Y. TAKAHASHI, *J. biol. Chem.* **235**, 1992 (1960).
16. J. AXELROD, R. J. WURTMAN and CH. M. WINGET, *Nature, Lond.* **201**, 1134 (1965).
17. W. M. McISAAC, G. FARRELL, R. G. TABORSKY and A. N. TAYLOR, *Science* **148**, 102 (1965).
18. F. MÄRKI, J. AXELROD and B. WITKOP, *Biochim. biophys. Acta* **58**, 367 (1962).
19. S. KVEDER, S. ISKRIĆ and D. KEGLEVIĆ, *Biochem. J.* **85**, 447 (1962).