BIOGENIC AMINES AND ACTIVE POLYPEPTIDES IN THE SKIN OF TEN JAPANESE AMPHIBIAN SPECIES

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Received for publication May 25, 1964

In the course of a systematic study on the constituents of amphibian skin active on vascular and extravascular plain muscle it soon became manifest that the skin of amphibians could represent an enormous store-house not only of biogenic amines (imidazole-, indole- and phenyl-alkylamines) but also of highly active polypeptides. The most extensive investigation on this topic has so far been carried out on South-American amphibian species (1–9). The present paper represents a first report of a more complete study we are planning to carry out on Asian amphibians.

MATERIALS AND METHODS

Amphibian material: The following amphibian species were collected during the late spring and summer 1962 (the number of specimens is given in parentheses): Rana japonica (10), Rana catesbiana (10), Rana limnocharis (21), Rana nigromaculata (10), Rana rugosa (17), Hyla arborea japonica (50), Triturus pyrrhogaster (20), Polypedatus buergeri (10), and Bufo vulgaris fumosus (10).

The fresh skins were carefully spread out and dried in the shade. In the case of Triturus it was impossible to separate the skin from the underlying skeleton. One gram dried skin corresponded to approximately 3.5–4.5 g fresh skin.

Ten fumigated specimens of Hymobius nobulosus (whole body, in trade as fine food) were also submitted to the same extraction procedure as the skin of the other amphibians. However, it is obvious that results obtained on this material are virtually of no value.

Extraction procedure: The dried skins were minced with scissors and then immersed in 8–10 parts (weight by volume) of 80% methanol. The liquid was decanted after a week, and the skins were re-extracted for another week with 6–3 parts of the same solvent. The methanol extracts were combined and filtered, and then kept in the refrigerator in dark bottles.

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Suitable amounts of the extracts were freed from methanol by distillation at 40-45°C immediately before use. In the case of paper chromatography the material was also treated, before application on paper, with petroleum ether, in order to remove fats.

**Paper chromatography**: After having been conveniently concentrated (1 ml liquid = 1-3 g dry skin), the extracts were submitted to ascending paper chromatography using Whatman no. 1 paper. The two following solvent mixtures were routinely employed: n-butanol : acetic acid : water (40 : 10 : 50) and n-butanol : 25% methylamine (80 : 30).

Paper chromatograms were sprayed with the following developing reagents: (a) 0.1-0.3% solution of Heinrich and Schuler NNCD reagent (2-chloro-1-nitro-1-diazo-β-α-naphthalene sulfonic acid) in 0.1 M HCl; (b) aqueous solution of diazotized sulfanilic acid (Pauly reagent) followed by 3-5% aqueous sodium carbonate; (c) aqueous solution of diazotized p-nitroaniline, followed by sodium carbonate; (d) 0.05-0.1% alcoholic solution of dichloroquinone chlorimide (Gibbs reagent) followed by sodium carbonate; (e) 1-2% alcoholic solution of p-dimethylaminobenzaldehyde, followed by exposure of the chromatograms to HCl vapours in a glass cabinet.

Semiquantitative estimation of biogenic amines on paper chromatograms was carried out by visual comparison of spots given by different known amounts of the standard synthetic amine with spots produced by different amounts of the skin extract.

**Bioassay**: The crude extracts of amphibian skin were assayed on the diuresis of hydrated rats and on the following muscle preparations: frog rectus abdominis, guinea-pig ileum, rabbit colon, rat colon and rat estrous-uterus. The frog rectus abdominis was used in the quantitative assay of leptodactyline, the rat uterus in the quantitative estimation of 5-hydroxytryptamine (5-HT). The same rat uterus and the other smooth muscle preparations were used in the detection, distinction and quantitative estimation of active polypeptides. For details on the bioassay procedures see preceding papers (2, 10, 11).

In order to eliminate, in the bioassay of polypeptides, the possible disturbing interference of indolealkylamines, histamine and acetylcholine, appropriate antagonists were added to the nutrient solution: atropine $10^{-7}$, mepyramine $10^{-7}$, and 2-bromolyserygic acid diethylamide $10^{-7}$ (BOL). On the other hand, in order to permit quantitative bioassay of 5-HT and other amines in the presence of active polypeptides, these were previously inactivated by digestion with chymotrypsin.

So far, about ten different active polypeptides have been detected in the amphibian skin, and their number is probably destined to increase. Research in progress is directed to the isolation of the different polypeptides and to the elucidation of their constitution and amino acid sequence. In the mean time it seems prudent to avoid premature identifications and classifications. This is even more imperative as very often amphibian skin contains a mixture of different active polypeptides interfering with each other in unexpected way.

However, at the present time two groups of polypeptides in the amphibian skin...
already seem to have characteristic features: the bradykinin-like polypeptides and the eledoisin-like polypeptides.

Bradykinin-like polypeptides may be considered, at least provisionally, those which, like bradykinin, (a) potently stimulate the guinea-pig ileum and even more the rat uterus, (b) depress the rat duodenum, (c) are inactive or nearly so on the rabbit colon, and (d) are inactivated by chymotrypsin, but are resistant to trypsin. Bradykinin-like polypeptides have so far been found in a number of Ranae and Phyllomedusae.

Eledoisin-like polypeptides are in their turn characterized by (a) a potent stimulant action on the rabbit large intestine and the guinea-pig ileum, (b) a potent hypotensive action in the anesthetized dog, (c) a negligible activity on the rat uterus, and finally (d) a complete inactivation both by chymotrypsin and trypsin. Eledoisin-like polypeptides have so far been detected in several Physalaemus and Phyllomedusa species.

It should be stressed that other polypeptides of the amphibian skin elude at present any definite classification.

Incubation with enzymes: Methanol extracts corresponding to 0.1-0.3 g dry skin were freed from the solvent and the residue dissolved in 0.9-1.8 ml isotonic NaCl adjusted to pH 7.4-7.7 with sodium carbonate. After adding 100 μg chymotrypsin or 1 mg trypsin in 0.1-0.2 ml distilled water, the liquid was incubated in a water bath for 30-60 min at 37°C. Enzymatic activity was stopped by immersion of the test tubes in a boiling water bath, for 3-5 min.

Standard synthetic compounds: The following synthetic compounds were used as standards: tryptamine hydrochloride, 5-hydroxytryptamine creatinine sulfate, N-methyl-5-hydroxytryptamine creatinine sulfate, bufotenine base, dehydrobufotenine (kindly supplied by Dr. Witkop, Bethesda), 5-hydroxyindoleacetic acid, 5-hydroxytryptophan, p-tyramine hydrochloride, leptodactyline picrate, candicine iodide, histamine dihydrochloride, noradrenaline base, bradykinin Sandoz, eledoisin and physalaemin Farmitalia.

RESULTS

1. Biogenic Amines

Table 1 shows that the only group of amines having a widespread distribution in the examined Japanese amphibian species is that of indolealkylamines. It is highly questionable whether the small stimulant effect dispayed on the frog rectus abdominis by the different extracts is attributable to leptodactyline. Similarly, histamine and related imidazolecalkylamines are either lacking or present in negligible amounts.

As expected, there is a variety of indolealkylamines in skin extracts of Bufo vulgaris formosus.

In addition to those of known amines, chromatograms may show more or less clear spots of unknown phenolic or indolic compounds. Chromatograms of Rana japonica, for example, show a 5-hydroxyindole spot having an Rf value slightly higher, both in the acid and in the alkaline solvent mixture, than that of 5-HT. Moreover, on nearly all chromatograms the Gibbs reagent causes the development of sky-blue coloured spots,
having the following Rf values in the butanol: acetic acid: water mixture: 0.20-0.25, 0.35-0.40, 0.60-0.70, and 0.85-0.9.

### Table 1. Amphibian skin. Content of biogenic amines (in \( \mu g \) free bases per g dry tissue).

<table>
<thead>
<tr>
<th></th>
<th>5-HT**</th>
<th>Other indole-alkylamines*</th>
<th>Histamine**</th>
<th>Leptodactyline**</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rana nigromaculata</em></td>
<td>70-80</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Rana catesbiana</em></td>
<td>&lt;1</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td><em>Rana japonica</em></td>
<td>60-70</td>
<td>unknown hydroxy-indole spot</td>
<td>&lt; 1</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td><em>Rana rugosa</em></td>
<td>750</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><em>Rana limnocharis</em></td>
<td>&lt;1</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td><em>Hyla arborea japonica</em></td>
<td>10</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><em>Polypedates buergeri</em></td>
<td>&lt;0.3</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td><em>Trinitrus pyrrhogaster</em></td>
<td>&lt;0.3</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><em>Hynobius nebulosus</em></td>
<td>&lt;0.2</td>
<td>n.d.</td>
<td>&lt; 1</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Bufo vulgaris formosus</em></td>
<td>80-100</td>
<td>N-methyl-5-HT 60</td>
<td>&lt; 1</td>
<td>1</td>
</tr>
</tbody>
</table>

*n.d. = not detectable (\(< 1-2 \mu g/g\)).

Buftotenidine has been expressed as bufotenine, bufothionine as dehydrobufotenine.

II. Polypeptides

Table 2 presents some data on the polypeptide content, expressed in terms of \( \mu g \) bradykinin or eledoisin per g dry tissue, of the skin of the examined Japanese amphi-

### Table 2. Amphibian skin. Polypeptide activities (expressed in terms of bradykinin or eledoisin) on different smooth muscle preparations.

<table>
<thead>
<tr>
<th></th>
<th>Rat uterus (bradyk. ( \mu g/g ))</th>
<th>Guinea-pig ileum (bradyk. ( \mu g/g ))</th>
<th>Rabbit colon (eledois. ( \mu g/g ))</th>
<th>Rat colon (eledois. ( \mu g/g ))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rana nigromaculata</em></td>
<td>10-12</td>
<td>16</td>
<td>0.1-0.2</td>
<td>3.0</td>
</tr>
<tr>
<td><em>Rana catesbiana</em></td>
<td>2.5</td>
<td>1.5</td>
<td>0.8-1.0</td>
<td>50</td>
</tr>
<tr>
<td><em>Rana japonica</em></td>
<td>1-2</td>
<td>2-3</td>
<td>0.05</td>
<td>2</td>
</tr>
<tr>
<td><em>Rana rugosa</em></td>
<td>80</td>
<td>110</td>
<td>0.1</td>
<td>60-70*</td>
</tr>
<tr>
<td><em>Rana limnocharis</em></td>
<td>0.2</td>
<td>&lt;1</td>
<td>&lt;0.05</td>
<td>&lt;1</td>
</tr>
<tr>
<td><em>Polypedates buergeri</em></td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0.1</td>
<td>&lt;1</td>
</tr>
<tr>
<td><em>Trinitrus pyrrhogaster</em></td>
<td>&lt;1</td>
<td>&lt;1.5</td>
<td>&lt;0.05</td>
<td>&lt;1</td>
</tr>
<tr>
<td><em>Hyla arborea japonica</em></td>
<td>0.1</td>
<td>3.4</td>
<td>&lt;0.1</td>
<td>&lt;2</td>
</tr>
<tr>
<td><em>Hynobius nebulosus</em></td>
<td>&lt;1</td>
<td>&lt;1.5</td>
<td>&lt;0.2</td>
<td>&lt;1</td>
</tr>
<tr>
<td><em>Bufo vulgaris formosus</em></td>
<td>&lt;2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;2</td>
</tr>
<tr>
<td><em>Rana esculenta</em></td>
<td>10-20</td>
<td>10-20</td>
<td>0.5-1</td>
<td>140</td>
</tr>
<tr>
<td><em>Rana temporaria</em></td>
<td>200-230</td>
<td>200-230</td>
<td>inhibition</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

*Threshold stimulant dose on the rat colon = 50\( \mu g \) dry skin of *R. catesbiana* per ml nutrient liquid.
bians and, for comparison, of two European frogs. It should be stressed here that provisionally we consider to be of polypeptide nature all substances the activity of which may be destroyed by incubation with chymotrypsin and eventually trypsin.

From the tabulated data it may be concluded that:

(a) None of the examined extracts contains detectable amounts of eledoisin-like polypeptides. In fact, stimulation of the rabbit large intestine is always extremely moderate and response differs qualitatively from that produced by eledoisin or physalaemin.

(b) With the possible exception of *Rana limnocharis*, all other species of *Rana* contain variable amounts of a bradykinin-like polypeptide which potently stimulates the rat uterus and, like bradykinin, is chymotrypsin-sensitive and trypsin-resistant. No nearer characterization of this polypeptide is possible at the present time, nor is it possible to decide whether it is the same in the different *Rana* species.

(c) Extracts of *Rana catesbiana* and *Rana rugosa* skin display a considerable stimulant activity on the rat colon which cannot be attributed either to a typical bradykinin-like polypeptide or to an eledoisin-like polypeptide. In fact bradykinin is inactive on this preparation or causes relaxation; eledoisin-like polypeptides in their turn may be excluded owing to the previously stressed inactivity of the extracts on the rabbit colon. It is quite possible that this unknown polypeptide is identical or similar to a polypeptide recently found, in larger amounts, in skin extracts of *Rana esculenta* (12).

(d) Extracts of the skin of the other examined amphibians are devoid of any appreciable polypeptide activity.

It should be added that with the exception of *Rana rugosa* and *Bufo formosus*, which were not taken into consideration owing to their high content of 5-HT and other anti-diuretic hydroxyindolealkylamines, none of the examined skin extracts show any important anti-diuretic activity when administered subcutaneously to hydrated rats in doses corresponding to 0.1 g dry skin per kg body weight.

Of course, all the above conclusions are valid for extracts of dry skin. It may well be that the bradykinin-like polypeptides found in the dry skin represent only a part of those occurring in the fresh skin, and it is evident that compounds completely incapable of withstanding the drying process may have escaped our attention.

**DISCUSSION**

Japanese amphibians behave like their European congeners in their content of biogenic amines and polypeptides.

Concerning biogenic amines the only ones found in the skin of the examined Japanese species are indolealkylamines. *Rana nigromaculata*, *Rana japonica*, *Rana rugosa* and *Hyla arborea japonica* contain only 5-HT, like the European *Rana esculenta* and *Hyla arborea*.

*Bufo formosus*, in its turn, presents the same spectrum of indolealkylamines as *Bufo bufo*. It has been shown, in Parma, that the European brown frogs (*Rana temporaria*, *Rana dalmatina*, *Rana latastei*) contain, in contrast to the common green frog *Rana esculenta*,
N-methylated 5-HT derivatives also. Accordingly, it would be worth while examining the Japanese *Rana temporaria*.

Recently Anastasi and co-workers (13) succeeded in demonstrating that the bradykinin-like polypeptide occurring in the skin of *Rana temporaria* is the nonapeptide Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, i.e. that it is identical with the bradykinin originating in mammalian blood by the action of proteolytic enzymes on bradykininogen. Research is in progress on the polypeptides of *Rana esculenta*. The results of these studies will probably contribute in a decisive manner to the solution of the problems related to the polypeptides occurring in the skin of Japanese frogs.

Similarly, experiments carried out in *Rana esculenta* and in other species in order to elucidate the physiological significance of the polypeptides in amphibian skin, may perhaps give results of general validity.

**SUMMARY**

Ten Japanese amphibian species were examined with regard to their content of biogenic amines and active polypeptides.

Three out of the five studied *Rana* species contained considerable amounts of 5-HT. This amine was also present in *Hyla arborea japonica* and, together with N-methyl-5-HT, bufotenine, bufotenidine, dehydrobufotenine and bufothionine in *Bufo formosus*.

Leptodactyline and histamine were either lacking or present in trace amounts in all the examined species.

The Japanese *Ranae* contained, like their European congeners, one or more typical bradykinin-like polypeptides and, sometimes, another polypeptide active on the rat colon. Eledoisin-like polypeptides were lacking.

**Acknowledgement** : This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Roma.

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