BIOGENIC AMINES AND ACTIVE POLYPEPTIDES OF THE AMPHIBIAN SKIN

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The purpose of this review article is to give an account of research carried out recently by our group and by other research-workers in the field of biogenic amines and active polypeptides of the amphibian skin.

Surprisingly enough, not only were biogenic amines found in the cutaneous tissue of amphibians the same as those occurring, in a much lesser variety and concentration, in mammalian tissues, but even active polypeptides of amphibian skin were often identical or very similar to those found in blood, brain, and gastrointestinal mucosa of mammals. Hence, results obtained in the study of amphibian skin may substantially contribute to the understanding and interpretation of results already obtained in mammals, and may offer the basis for new research trends in higher vertebrates.

It is therefore hoped that the present article may be of interest also to people other than those cultivating comparative pharmacology and biochemistry.

AMINES

Three groups of aromatic amines will be considered successively: indolealkylamines, imidazolealkylamines, and hydroxyphenylakylamines.

Indolealkylamines.—Indolealkylamines represent the longest known and most thoroughly investigated group of aromatic amines of the amphibian skin. The chemical structure of some representatives of this group has been elucidated by Wieland and co-workers since 1930–1931 (cf. 1).

Erspamer & Vialli (2) first described the occurrence of 5-hydroxytryptamine (5-HT) in the amphibian skin and pure 5-HT was isolated from skin extracts of *Discoglossus pictus* (3). Since then, the content of indolealkylamines has been examined in the skin of more than 300 amphibian species collected throughout the world, and this study has led to a considerable extension and confirmation of the preceding results and to the acquiring of a number of new data, among which is the identification of indolealkylamines previously unknown in nature (4–11).

It was shown that 5-HT had a very wide distribution in the amphibian skin, being present in the majority of the examined genera and families. Similarly, well represented were the N-methylated derivatives of 5-HT: N-

methyl-5-HT, bufotenine, and bufotenidine. The last two compounds were found not only in toads (whence their name) but also in several other genera and families (*Rana, Leptodactylus, Hyla, Nictimystes, Xenopus* etc.), including representatives of urodeles. Neither dehydrobufotenine was peculiar to toads, since it was detected, in small amounts, in the skin of *Leptodactylus pentadactylus dengleri.*

The skin of *Bufo alvarius* contained large quantities of 5-methoxylated tryptamines, represented by 5-methoxy-N-methyltryptamine and 5-methoxy-N,N-dimetryltryptamine or O-methylbufotenine. Previously, 5-methoxytryptamines were known to be present in the pineal body, in the form of melatonin or 5-methoxy-N-acetyltryptamine, and in some South American vegetables (12).

Up to present studies the only known example of conjugated indolealkylamines in the amphibian skin was that of bufothionine, the O-sulphate of dehydrobufotenine. In mammalian urine two O-conjugates of 5-HT have been described, the O-sulphate of 5-HT and the O-glucuronide of 5-HT, which are to be considered normal metabolites of the amine (13).

Now a list of six or seven conjugated tryptamines, all with sulfuric acid, is available for the amphibian skin and, what seems to be more important, it has been found that sulfuric acid may be attached not only to the phenolic —OH group but also to the pyrrolic >NH group.

There is strong evidence that the following sulfoconjugates occur in the skin of toads: O-sulfate of 5-HT (*Bufo regularis* and other African toads), O-sulfate of bufotenine (*Bufo spinulosus chilensis, Bufo bocourti, Bufo calamita,* etc.), O-sulfate of dehydrobufotenine (numerous species of toads), bufotenine 1-sulfonic acid or bufoviridine (*Bufo viridis, Bufo calamita, Bufo debilis, Bufo alvarius, Bufo koynayensis,* etc.), O-methylbufotenine 1-sulfonic acid or O-methylbufoviridine (*Bufo alvarius*), and finally tryptamine 1-sulfonic acid (*Bufo carens*). Of interest is that the O-sulfate of bufotenine has been recently detected, in large amounts, also outside the genus *Bufo,* in the skin of the Australian *Hyla pearsoniana.*
amphibian, *Nictimystes tympanocryptis*, the occurrence of the O-sulfate of bufotenidine is probable (14).

African toads and *Bufo alvarius* contained in their skin several other indole derivatives, the identification of which is in progress. It is possible that some of them differ conspicuously from the hitherto known indole compounds.

Among the metabolites produced by the action of monoamineoxidase on the tryptamines, the following have been traced, especially in extracts of dried skins: indoleacetic acid, 5-hydroxyindoleacetic acid, 5-methoxyindoleacetic acid, 5-hydroxytryptophol, and 5-methoxytryptophol.

The content of indolealkylamines in the amphibian skin ranged from a few micrograms to as much as 100–150 milligrams per g dry tissue (glands of *Bufo alvarius*).

Figure 1 gives a synopsis of the different indolealkylamines found in the skin of amphibians and indicates the enzyme systems involved in their biosynthesis. Necessary intermediates not yet traced are in parentheses. Only the most important formulae are reproduced.

The pharmacological actions of indolealkylamines are described and discussed in detail in monographs (15, 16) and symposia (17).

Imidazolealkylamines.—One of the most interesting results of the screening of amphibian skin for biogenic amines was the discovery, in skin extracts of the South American frog *Leptodactylus pentadactylus labyrinthicus,* of a surprisingly rich series of imidazolealkylamines, some hitherto unknown in nature, and others of rare occurrence (8, 18, 19).

Figure 2 represents the imidazole derivatives present in the amphibian skin, together with their possible biosynthetic correlations. In parentheses are intermediates as yet not detected in the skin.

Spinacine is an amino acid found by Ackermann & Müller (20, 21) in the liver of the shark *Acanthias vulgaris* and in the tissues of the crab *Crango vulgaris*. However, a derivation of spinaceamine from spinacine is hardly conceivable, because attempts to decarboxylate the amino acid in vitro have so far been unsuccessful.

Another derivative of spinaceamine, 6,7-dimethylspinaceamine, has been detected in acetone extracts of the skin of *Leptodactylus pentadactylus labyrinthicus* and, even in larger amounts, in *Leptodactylus laticeps*. The compound does not appear in Figure 2 because the problem is still open whether the spinaceamine derivative actually pre-exists in the skin or is an artifact of acetone extraction.

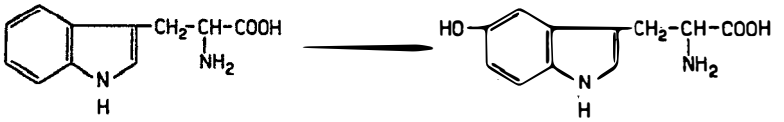
As far as histamine is concerned, large amounts of it are present not only in different species of the "pachypus" group of the genus *Leptydactylus,* but also in several Australian species of *Hyla* and *Taudactylus* (14).

N-Acetylhistidine is contained in the amphibian skin in small amounts, whereas it is present in very high concentrations (up to 1000 μg and more per g fresh tissue) in the brain, eye, and heart of the same amphibians and of other piokilothermal vertebrates (22).

To give a quantitative idea of the content of imidazole derivatives in the amphibian skin, the following data are reported concerning *Leptodactylus pentadactylus labyrinthicus*. Values are in μg of free bases per g dry tissue: histamine, 50–740; N-methylhistamine, 110–740; N,N-dimethylhistamine, 35–230; N-acetylhistamine, 2–15; spinaceamine, 6–120; 6-methylspinaceamine, 50–410; 6,7-dimethylspinaceamine(?), 10–160; histidine, 5–20; N-acetylhistidine, 5–10.

As in the case of indolealkylamines, the assortment of imidazolealkylamines occurring in the *Leptodactylus* skin is the result of the activity of a set of enzymes: histidindecarboxylase(s), imidazole N-methyltransferase (imidazole) N-acetylase and (imidazole) cyclising enzymes.

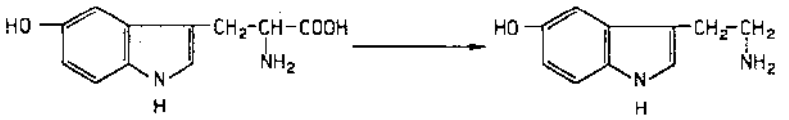
Trytptophan 5-Hydroxylase



Tryptophan 5-Hydroxytryptophan

Aromatic L-Amino Acid Decarboxylase

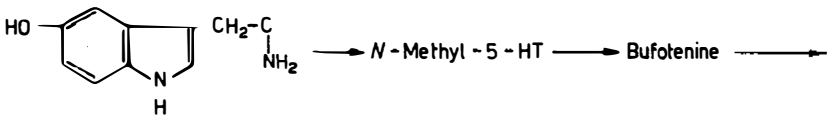
(5-Hydroxytryptophan Decarboxylase)



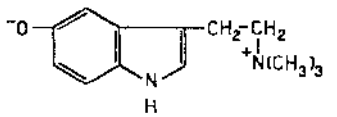
5-Hydroxytryptophan 5-Hydroxytryptamine

Tryptophan → Tryptamine

N-Methyltransferase



5-Hydroxytryptamine (5-HT)



Bufotenidine

[5-Methoxytryptamine (5-MT)] → *N*-Methyl-5-MT → *N*,*N*-Dimethyl-5-MT

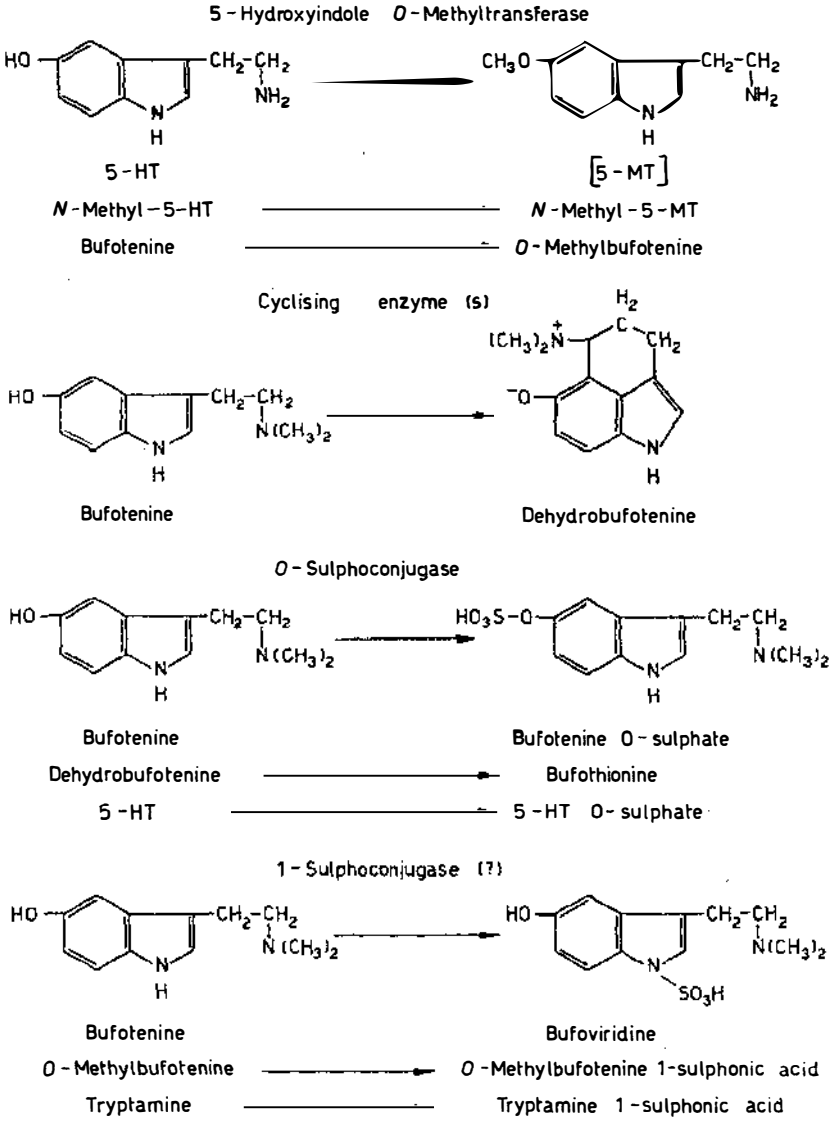


FIG. 1. Enzyme systems involved in the biosynthesis of the indolealkylamines occurring in the amphibian skin. Parentheses indicate compounds not yet found in the skin.

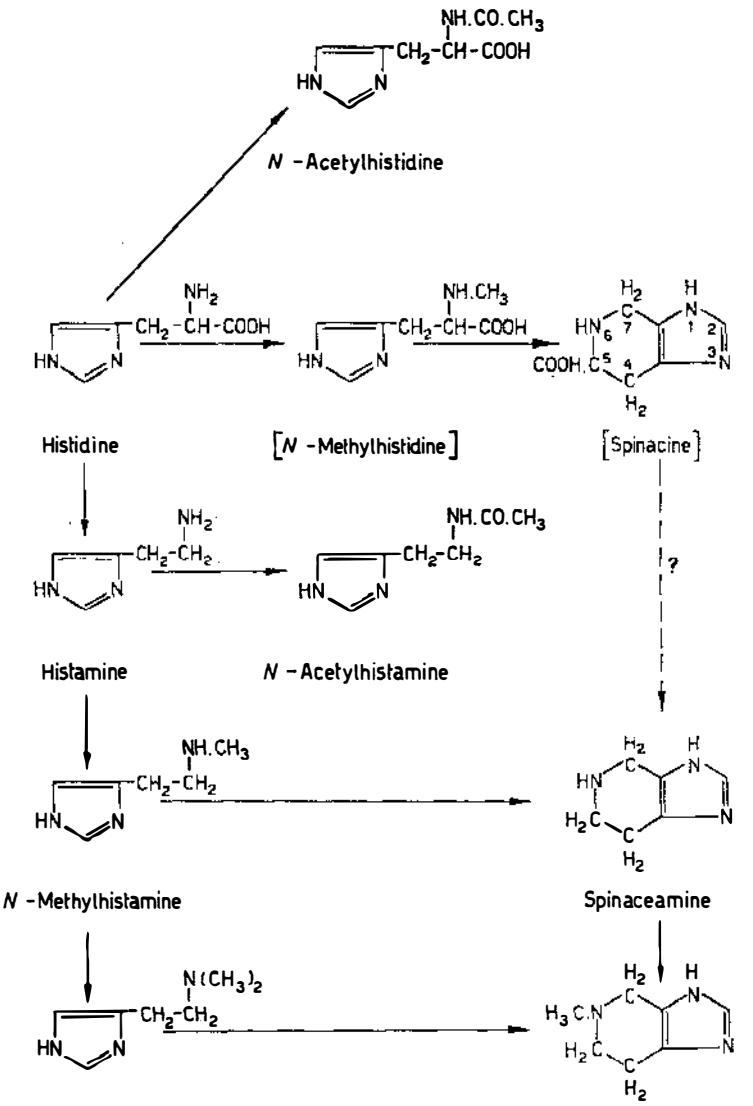


FIG. 2. Biosynthetic pathways for imidazolealkylamines of the amphibian skin. Parentheses indicate compounds not yet found in the skin.

Histamine is a good substrate for monoamineoxidase and for diamineoxidase. Thus, small amounts of imidazoleacetic acid may be found, together with histamine, especially in extracts of dried skin.

It is generally stated that methylation of the ring nitrogen(1-methylation) represents the main metabolic route of histamine, and methylation of the side chain (N-methylation) represents only a minor metabolic route. This statement, which cannot certainly be accepted for the amphibian skin, should be re-examined also for the mammalian organism. In fact, Navert et al (23) have recently demonstrated that following infusion of histamine(2-ring-^{14}C) dihydrochloride, N-methylhistamines were predominant not only in gastric juice, but also in aortic and portal plasma of the dog, and were excreted in the urine in the same amount as 1,4-methylhistamine.

Moreover, 1-methylation and N-methylation should be considered under a different perspective, insofar as the first is a route of actual inactivation of histamine, the second a metabolic pathway leading to derivatives that in every case retain conspicuous histamine-like activities and may even be, on particular receptor sites, more active than histamine itself. It seems possible, for instance, that N,N-dimethylhistamine is the preferred specific derivative of histamine for stimulating HCl secretion in the dog stomach (24).

For the pharmacology of histamine and its derivatives the reader is referred to a recent monograph on the topic (25). A relevant pharmacological study on N-methylhistamines and spinaceamines has been carried out by Bertaccini & Vitali (26).

Hydroxyphenylalkylamines.—The occurrence of varying amounts of epinephrine and norepinephrine (up to 2–5% of the total catecholamine content) has been described in *Bufo marinus, Bufo bufo,* and other amphibian species. In *Bufo bufo* the concentration of epinephrine was 3.7 μg and that of norepinephrine 0.2 μg per g fresh skin (1, 27).

Catecholamines were never traced in our paper chromatographic and biological screening, possibly owing to inadequate extraction and purification procedures, and because very often only dried skins were available for study. However, research was successful for the more stable monohydroxyphenylalkylamines.

Several South American amphibian species belonging to the superfamily *Leptodactilidae* contained in their skin *m*-hydroxyphenylethyltrimethylammonium, the quarternary ammonium derivative of *m*-tyramine. This was called leptodactyline, since it was first found in, and isolated from the skin of *Leptodactylus ocellatus,* where it may reach concentrations up to 9000 μg per g dry skin (28).

The *para* analog of leptodactyline, candicine, first described in some Argentinian *Cactaceae,* has been traced, together with the corresponding primary amine, *p*-tyramine, in the skin of *Leptodactylus pentadactylus pentadactylus* (29).

Other phenolic spots have been developed by suitable spraying reagents on chromatograms of extracts of amphibian skin. Some of them are certainly composed of amines (*Bufo kisoloensis, Melanophryniscus moreirae*), others of phenolic acids, still others of amino acids or polypeptides.

The biochemical correlations existing between the hydroxyphenylalkylamines occurring in amphibian skin are represented in Figure 3. Intermediates not yet found in the skin are in parentheses.

From the above data the activity of the following enzyme systems must be postulated: phenylalanine hydroxylase(s), capable of hydroxylating the phenylalanine molecule at position 4 (*para*), at position 3 (*meta*), or at both positions; aromatic acid decarboxylase(s), responsible for the decarboxylation of the tyrosines and of dopa; a potent phenylethylamine N-methyltransferase causing a rapid transformation of the primary amines into the secondary amines (epinephrine) or the quaternary ammonium bases (candicine and leptodactyline). N-Methylation is apparently most vigorous for *m*-tyramine, since neither *m*-tyramine itself nor the corresponding secondary or tertiary amine could be traced in amphibian skin. In the case of norepinephrine and epinephrine also the occurrence of a dopamine β-hydroxylase must be postulated.

Leptodactyline displayed, in vertebrates, powerful nicotinic actions and manifested a marked neuromuscular blocking effect of the depolarizing type. The nicotinic action was especially evident: (*a*) on the dog and cat blood pressure which, following intravenous injection of leptodactyline (threshold dose 1–5 μg/kg) presented a sharp rise, completely antagonized by ganglionic blocking agents; (*b*) on the isolated guinea-pig and rabbit atria, on which the base exerted positive inotropic and chronotropic effects before atropinization and exactly opposite effects after atropinization; (*c*) on respiration, which was transiently stimulated, via the carotid chemoreceptors; finally (*d*) on the frog rectus abdominis muscle which was contracted at concentrations of the base as low as 0.01–0.03 μg per ml nutrient liquid. On this preparation, leptodactyline was 4–5 times more potent than acetylcholine and 50–100 times more potent than nicotine.

On the sciatic nerve-gastrocnemius muscle preparation of the dog and the cat a nearly complete twitch inhibition was obtained with 100 μg/kg leptodactyline, intravenously (30).

Candicine possessed barely 5–10% of the activity of leptodactyline. Several synthetic leptodactyline-like compounds were studied in comparison with leptodactyline (31).

It is possible that extracts from *Leptodactylus* skin have been used by the natives of South America, together with other amphibian extracts, in the preparation of some "curares."

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The turnover rate of biogenic amines in the amphibian skin is not known. However, some indications may come from preliminary experiments

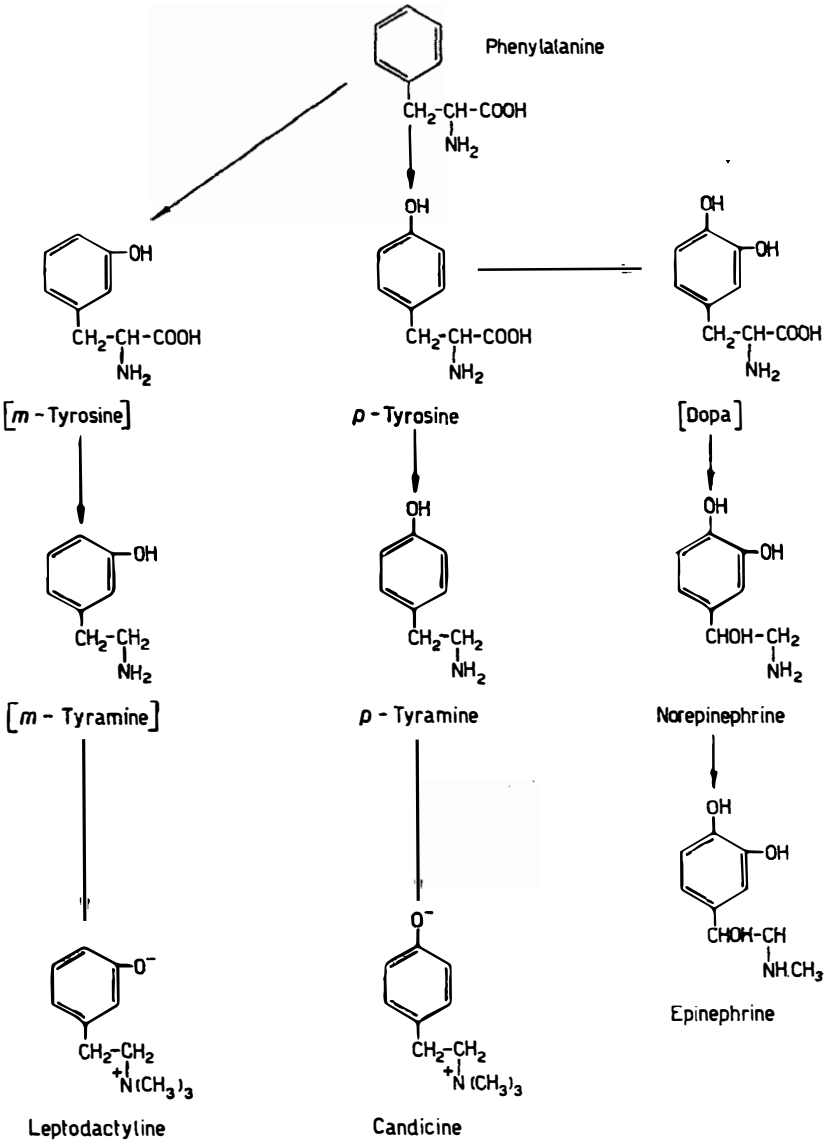

FIG. 3. Biosynthetic pathways for phenylalkylamines of the amphibian skin. Parentheses indicate compounds not yet found in the skin.

on post-mortal biosynthesis of amines in the cutaneous tissue, carried out on fresh and dried moieties of individual skins. Occasionally, a considerable increase in the amine content was observed during drying of the skin of *Leptodactylus pentadactylus labyrinthicus* and *Hyla caerulea,* up to 50–150 μg of 5-HT and histamine per g wet tissue. Since enzyme activity during drying is probably limited to the first 12–24 hours, the observed amine increase would denote a turnover rate of considerably greater intensity than suspected.

For the indolealkylamines and phenylalkylamines the necessity has been postulated of the occurrence in the skin of enzymes capable of hydroxylating the aromatic nucleus; similarly for all three groups of amines herein considered the necessary occurrence of N-methyltransferases has been demonstrated. Now the problem arises of whether each of the above biosynthetic processes is carried out by a single enzyme or by multiple enzymes, depending on the substrate.

Whereas different enzymes must be admitted for hydroxylating processes, things are not so clear for processes involving transfer of methyl groups to the amine nitrogen of the lateral chain. However, it is evident that the fact that in the presence of both 5-HT and histamine the skin of *Leptodactylus pentadactylus dengleri* or *Leptodactylus vilarsi* is capable of N-methylating only 5-HT, and the skin of *Leptodactylus pentadactylus labyrinthicus* only histamine, is in favor of the activity of different N-methyltransferases.

A similar problem must be raised for the sulfoconjugases which may transfer a sulfuric acid radical either to the phenolic —OH group or to the pyrrolic >NH group of the indolealkylamines. Sometimes both conjugates occur in the same skin (O-sulfate of bufotenine and bufotenine 1-sulfonic acid in *Bufo alvarius* and *Bufo calamita,* O-sulfate of dehydrobufotenine and bufotenine 1-sulfonic acid in *Bufo punctatus*), but in other instances only one of the two conjugates is present in the skin (O-sulfate of dehydrobufotenine in *Bufo bufo bufo* and numerous other toads; O-sulfate of bufotenine in *Bufo spinulosus chilensis, Bufo bocourti, Hyla pearsoniana;* bufotenine 1-sulfonic acid in *Bufo debilis, Bufo koynayensis*). These observations again point to a plurality rather than to a oneness of sulfoconjugases.

A number of amines of the amphibian skin (cyclised and conjugated amines) have been found to be poorly active or even completely inactive on blood pressure, respiration, and the usual smooth muscle preparations. However, it might well be that these compounds are active on test systems others than those checked, or may influence some biochemical events as yet unknown.

The study of biogenic amines in amphibian skin has been pursued in this laboratory with determination because we are persuaded that data obtained in amphibians are largely valid also for mammals. Amphibian skin, owing to the variety and abundance of amines contained in it, is a material extraordinarily suitable for the detection of new amines and new metabolic path-

ways. Virtually every theoretically conceivable amine derivative may be traced in amphibian skin. It is only a matter of screening more and more new species. What is not present in one species is present in another; what has been lacking in 300 species may be found in the 301st one.

It is obvious that once the possibility of the biosynthesis of a new amine or metabolite is demonstrated in amphibian skin and the new compound has been characterized with sufficient accuracy, it will be much easier to check its presence also in mammalian tissues or urines, under normal and pathological conditions. Similarly, it will be easier to achieve a more complete identification of the metabolites of exogenous amines. Some of the hitherto "unknown" indole, imidazole, or phenolic spots found on chromatograms of tissue or urine extracts will then be recognized and exactly interpreted.

In a few words, we have been and are considering the amphibian skin as a *locus minoris resistentiae* for elucidating problems of general significance.

POLYPEPTIDES

The active polypeptides so far detected in the amphibian skin may be divided into five groups characterized by distinctive features: eledoisin-like polypeptides or tachykinins, bradykinin-like polypeptides or bradykinins, caerulein-like polypeptides, alytesin-like polypeptides, and finally, miscellaneous polypeptides, a store-group in which those peptides are provisionally placed which still await full elucidation of their structure or a sufficiently complete pharmacological study.

Eledoisin-like polypeptides.—The members of this polypeptide group display pharmacological actions mimicking those of eledoisin, an endecapeptide first found in the posterior salivary glands of *Eledone moschata,* a Mediterranean octopod (32–35).

The most important eledoisin-like polypeptide detected in amphibian skin is physalaemin, which has been isolated in a pure form from methanol extracts of the skin of *Physalaemus bigilonigerus* and which is present in skin extracts of other *Physalaemus* species as well (*Physalaemus centralis, Physalaemus bresslaui*). The content of physalaemin ranged between 370 and 700 μg per g dry skin. No important losses of the polypeptide occurred during drying of the skin and recovery of the pure polypeptide from crude extracts was satisfactory (36).

Like eledoisin, physalaemin displayed a tremendously intense hypotensive action in the dog (threshold intravenous dose, 0.1–0.5 ng/kg), a potent stimulant action on the isolated large intestine of the rabbit (threshold 0.2–1 ng/ml), and the isolated ileum and large intestine of the guinea-pig, while eliciting a poor response in the rat uterus and rat colon (37, 38). Moreover, the polypeptide produced a powerful stimulation of the salivary and lacrimal secretions of the chicken, rat, dog, and man (39–44) and strikingly increased capillary permeability in the guinea-pig, rat, and man (45). When assayed on the vascular bed of the hind limb musculature of the dog, physa-

laemin, given by close intraarterial injection, was about 50 and 30,000 times more active than eledoisin and nitroglycerin, respectively. The minimal dose active on arteria poplitea flow was less than one picogram (46).

On the in situ jejunal loop of the anaesthetized dog physalaemin was twice as potent as cholecystokinin-pancreozymin, on a molar basis, 15 times as potent as human gastrin I, 50–100 times as potent as either bradykinin or carbachol, finally, more than 300 times as potent as acetylcholine, eserine, histamine, vasopressin, and 5-HT. Only caerulein overcame physalaemin in its stimulant effect, by three times (47).

In clinical trial physalaemin gave apparently satisfactory results in the treatment of the Sjögren syndrome (48).

It has been known for several years that skin extracts of the South American hylid frogs of the genus *Phyllomedusa* displayed physalaemin-like activities. The polypeptide or, more likely, one of the polypeptides responsible for this action, has now been isolated from the skin of *Phyllomedusa bicolor* and its structure has been fully elucidated (49). From the formulae reported below it may be seen that in its amino acid composition and sequence phyllomedusin is strictly related both to eledoisin and to physalaemin, the C-terminal pentapeptide being identical to that of eledoisin, the N-terminal pentapeptide more similar to that of physalaemin. Phyllomedusin displayed the same spectrum of activity as physalaemin with some quantitative differences (50).

In their biological and chemical features physalaemin and phyllomedusin are very similar not only to eledoisin, as already stated, but also to substance P, a polypeptide or, more likely, a family of polypeptides, found in the brain and gastrointestinal wall of vertebrates.

Physalaemin: Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2
Eledoisin: Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Phyllomedusin: Pyr–Asn–Pro–Asn–Arg–Phe–Ile–Gly–Leu–Met–NH_2
Substance P: Glu, Pro, Ser, Lys, Asp, Ala, Phe, Ile, Gly, Leu, Met plus other 10 to 16 amino acid residues

In fact, it may be seen that all the eleven amino acid residues of eledoisin are present also in substance P (51, 52). From studies carried out in this and other laboratories on more than 100 eledoisin- and physalaemin-like polypeptides it may be reasonably expected that the C-terminal tripeptide of substance P is -Gly-Leu-Met-NH_2 and that the phenylalanine residue occupies position 5 from the C-terminus.

Substance P shares with eledoisin and physalaemin the same activity spectrum and the three peptides can hardly be distinguished from each other even in parallel bioassay (53). Hence, results obtained with one of these peptides are presumably valid also for the other two, and eledoisin or physalaemin can be profitably used as model polypeptides in studies intended to elucidate the possible physiological significance of substance P in brain and intestine.

Physalaemin and phyllomedusin are not the only representatives of eledoisin-like polypeptides occurring in nature. Another highly active peptide belonging to this group is uperolein from the skin of the Australia amphibian *Uperoleia rugosa* (54). Still other physalaemin-like peptides probably occur in the skin of the Australian amphibians *Taudactylus* and *Pseudophryne,* and in the skin of African amphibians.

Eledosin, physalaemin, phyllomedusin, substance P, and related polypeptides are characterized by a prompt stimulant action on extravascular smooth muscle and a prompt hypotensive action. For this reason it has been suggested to attribute to this polypeptide group the denomination of *tachykinins,* as opposed to the group of slow-acting kinins, the true bradykinins.

Bradykinin and bradykinin-like polypeptides.—Polypeptides of this group were characterized by a remarkable but not exceptionally intense hypotensive action in the dog, rabbit, and cat; by a potent stimulant action on the isolated guinea-pig ileum and cat large intestine; by a formidable stimulant action on the estrous rat uterus. They have a poor stimulant action on the rabbit and rat colon and display an inhibitory action on the rat duodenum. Bradykinin-like polypeptides effectively increase capillary permeability in man and experimental animals and cause pain when administered intraarterially or intraperitoneally. All the known bradykinins are completely inactivated by incubation with chymotrypsin but are resistant to trypsin.

In addition to authentic bradykinin (I), 7 natural bradykinin-like polypeptides have been so far described: kallidin or lysyl-bradykinin(II), methionyl-lysyl-bradykinin or methionyl-kallidin(III), glycyl-bradykinin(IV), bradykinyl-isoleucyl-tyrosine-O-sulphate or phyllokinin(V), polystes-kinin (VI), and finally colostrokinin(VIII).

(I) Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(II) Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(III) Met–Lys–ArgPro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(IV) Gly–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(V) Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–Ile–Tyr(SO_3H)
(VI) Pyr–Thr–Asp–Lys–Lys–Leu–Arg–Gly–Bradykinin
(VII) Val–Pro–Pro–Gly–Phe–Thr–Pro–Phe–Arg
(VIII) Phe–, Arg, Asp, Glu_2, Gly, His, Ile, Leu_2, Lys_2, Pro_2, Val, –Ser
(IX) Bradykinyl–Val–Ala–Pro–Ala–Ser

Bradykinins (I), (II), and (III) occur in mammalian plasma, colostrokinin (IX) in colostrum, bradykinins (IV) and VI) in the venom of *Polystes* wasps (55), finally bradykinins (I), (V), and (VII) in the amphibian skin, together with the peptide (IX) which may be considered a bradykinin precursor.

Authentic bradykinin (I) has been isolated from the skin of the common European brown frog *Rana temporaria* in which it is contained in amounts as high as 200 to 250 μg per g fresh tissue (56).

Phyllokinin (V) has been prepared in a pure form from skin extracts of the Brazilian frog *Phyllomedusa rohdei* (57) and is probably present also in *Phyllomedusa bicolor* and in other *Phyllomedusa* species as well. It is the first and so far the only active natural bradykinin-like peptide with amino acid residues attached at the C-terminus of the bradykinin molecule. The activity spectrum of phyllokinin is similar to that of bradykinin, in comparison, to which it is 7–350% as potent, depending on test preparations (58).

Val^{1}-Thr^{6}-bradykinin (VII) has been isolated from extracts of the skin of the Japanese frog *Rana nigromaculosa,* together with authentic bradykinin and polypeptide (IX). Its stimulant activity on the rat uterus was about ⅕ of that of bradykinin (59).

Bradykinin or bradykinin-like peptides occur in the skin of several other species of the genus *Rana* and, outside the genera *Phyllomedusa* and *Rana,* in the skin of other amphibian species as well (e.g. *Ascaphus truei*) (60).

Colostrokinin has been recently obtained in a pure form from bovine colostrum. It is composed of 17 amino acid residues, the N-terminal residue being phenylalanine and the C-terminal residue serine. The position of the other amino acid residues remains to be established. On the dog blood pressure colostrokinin had 25% of the activity of bradykinin and on the rat uterus barely 3–6%. However, colostrokinin was 2–2.5 times more potent than bradykinin on the capillary permeability of the guinea-pig (61).

Caerulein and caerulein-like polypeptides.—Caerulein is a decapeptide first isolated from methanol extracts of the skin of the Australian hylid frog *Hyla caerulea,* where it was present in concentrations of 100–1000 μg per g fresh skin (62). Later on, authentic caerulein was prepared from the skin of the South American leptodactylid frog *Leptodactylus pentadactylus labyrinthicus* and of the South African amphibian *Xenopus laevis* (300–800 μg/g fresh skin) (63). It is probable that caerulein is present also in the skin of a number of other Australian hylid frogs (in *Hyla infrafrenata* and *Hyla moorei* up to 2500–3000 μg per g dry tissue) and South American leptodactylis frogs (in *Leptodactylus laticeps* up to 1300 μg per g fresh tissue).

The skin of the South American hylid frogs of the genus *Phyllomedusa* contained, in its turn, phyllocaerulein, a nonapeptide strictly related to caerulein. In *Phyllomedusa sauvagei,* whence it has been isolated in a pure form, it was present in amounts of 200–650 μg per g fresh skin (64).

Caerulein soon revealed a tremendous stimulant action on the musculature of the gall bladder and small intestine and a formidable stimulant action on the pancreatic secretion, together with a conspicuous stimulant action on gastric secretion.

The formulae below, showing the close chemical resemblance existing between the caeruleins, on the one side, and the gastrins and cholecystokinin-pancreozymin on the other side, explain the activity spectrum of the

caeruleins. It may be seen that caerulein has in common with human gastrin II the C-terminal pentapeptide and the sulphated tyrosyl residue, and with cholecystokinin-pancreozymin the entire C-terminal octapeptide, with the only unimportant difference of a methionyl residue substituted for the threonyl residue at the 6 position from the C-terminus.

Pyr–Gln–Asp–Tyr(SO_3H)–Thr–Gly–Trp–Met–Asp–Phe–NH_2 Caerulein
Pyr–Glu–Tyr(SO_3H)–Thr–Gly–Trp–Met–Asp–Phe–NH_2 Phyllocaerulein
Pyr–Gly–Pro–Trp–Leu–$(Glu)_5$–Ala–Tyr(SO_3H)–Gly–Trp–Met–Asp–Phe–NH_2 Human gastrin II
–Asp–Tyr(SO_3H)–Met–Gly–Trp–Met–Asp–Phe–NH_2 C-terminal octapeptide of cholecystokinin–pancreozymin

Extensive pharmacological investigation has shown that caerulein (the same is valid for phyllocaerulein) possessed exactly the same activity spectrum as cholecystokinin-pancreozymin, together with conspicuous gastrin-like effects.

The main pharmacological actions of caerulein may be summarized as follows:

(*a*) Potent spasmogenic action on the in vivo and in vitro gall bladder musculature of all the tested animals, including man. In vivo threshold doses were of the order of 0.15–1 ng/kg/min by intravenous infusion and of 0.01–0.1 μ/kg by subcutaneous administration. The effect was atropine-resistant (65–68).

(*b*) Potent stimulant action on the in situ musculature of the gut, with the possible exception of the duodenum, at least in man. The threshold dose by intravenous infusion was 0.5–1 ng/kg/min. Some isolated preparations of intestinal smooth muscle were similarly extremely sensitive to caerulein ($<$ 1 ng/ml of nutrient liquid). Atropine reduced the spasmogenic effect of caerulein (69–71).

(*c*) Relaxing action on the coledocho-duodenal junction (65, 72).

(*d*) Potent stimulant action on the exocrine pancreas causing the secretion of abundant pancreatic juice rich in enzymes (73–75). In dogs provided with chronic pancreatic fistulas the dose of caerulein required for 50% of maximal response was 0.5–0.7 ng/kg/min (74). In acute experiments in the dog the infusion of caerulein was capable of maintaining an intense, constant flow of pancreatic juice up to 10–20 hr (73). Repeated subcutaneous administration of caerulein caused a remarkable increase (up to 100%) of amylase and chymotrypsin concentration in the rat pancreas and a 80–100% increase in the incorporation of ^{14}C-leucine into protein by pancreas tissue slices of fasted guinea-pigs (76). In chickens the threshold dose of caerulein active on pancreatic secretion was 0.1–0.3 ng/kg/min. Maximal observed increases were 8-fold for volume output, 23-fold for amylase output, 18-fold for lipase output, and 28-fold for output of total tryptic activity. The concentration of enzymes in caerulein-juice was 3- to 6-fold the concentration in control-juice (88).

The pancreatic islets, too, were stimulated by doses of caerulein of the same order of magnitude as those active on the exocrine pancreas. In fact, in the dog, doses of the polypeptide as low as 2 ng/kg/min produced a 2.5- to 4-fold increase of the immunoreactive insulin levels in pancreatico-duodenal venous blood and a 3.5 -fold increase of the immunoreactive glucagon levels. The effect lasted as long as the infusion was continued (78, 79).

(*e*) Conspicuous stimulant action on the Brunner glands (80).

(*f*) Potent stimulant action on gastric secretion, with increase in volume, acid, and pepsin outputs. Concentration of hydrochloric acid in the dog juice increased up to 50%, and concentration of pepsin up to 250% (81).

In dogs with denervated gastric pouches the dose of caerulein needed to produce one-half of the maximal acid secretion was 8 ng/kg/min; in gastric fistula dogs 2.7 ng/kg/min (82). In man the threshold intramuscular dose active on volume and acid output was 50 ng/kg, and the optimum dose 250 ng/kg (83). Histamine- and pentagastrin-induced gastric secretion was inhibited by caerulein (84, 85). In its turn, the effect of caerulein was completely abolished by atropine in the dog, man, and chicken, but was atropine-resistant in the rat (77, 81, 83).

In the rat, caerulein caused also a remarkable increase in the secretion of the intrinsic factor. With 0.5 μg/kg of the polypeptide given subcutaneously the increase in intrinsic factor secretion was 100%; with 5 μg/kg, 350%. By intravenous infusion the threshold dose of caerulein was 10 ng/kg/min. On a molar basis caerulein was more than 10,000 times as active as histamine (86).

Finally, always in the rat, caerulein produced an increase in the histidine decarboxylase activity of the gastric mucosa. The threshold dose for a 3-hour infusion period appeared to be 0.5 μg/kg/hr. With 5 μg/kg/hr the enzyme activity was increased by 400% (87).

(*g*) Very moderate effect on flow of hepatic bile in the rat, but potent effect in the chicken and the dog.

In the chicken (88) the intravenous infusion of the caerulein elicited the following effects: (*i*) increase in volume of bile flow. The threshold dose of the polypeptide was 0.5 ng/kg/min and maximum increase of bile flow was 8-fold; (*ii*) increase in output of bile salts, cholesterol, pigments, and bicarbonate. At an infusion rate of 0.5 ng/kg/min the output of bile salts increased by 25% and that of cholesterol by 75%. Except for bicarbonate, the concentration of the bile components was higher in the caerulein bile than in control bile; (*iii*) increase in the excretion rate of exogenous bile salts, with removal of the autoinhibition produced by infusion of large amounts of these salts. With 15 ng/kg/min increase was 5-fold; (*iv*) acceleration of the transhepatic transport of sulphobromophthalein with simultaneous increase in plasma BSP clearance (threshold 1 ng/kg/min caerulein); (*v*) acceleration of the transhepatic transport of rose Bengal and indocianine, with contemporaneous increase in the secretion of endogenous bile salts.

In the dog (89) half of the maximal calculated increase in bile flow was

produced by 0.25 ng/kg/min of caerulein. Caerulein-bile showed an increased concentration in bicarbonate and chloride but not in bile salts. The output of these salts was not significantly different from control values after infusion of caerulein at rates ranging from 0.1 to 12.5 ng/kg/min.

(*h*) Potent stimulant effect on calcitonin release from porcine thyroid, perfused in situ. Approximately 1.3 ng/ml of caerulein doubled the secretion rate of calcitonin (90).

(*i*) Moderate action on the systemic blood pressure. In dogs and rabbits caerulein nearly always produced hypotension with a good dose/response relationship. In the dog the threshold dose was 10–100 ng/kg by intravenous injection, 5–15 ng/kg/min by intravenous infusion, and 5–10 μg/kg by subcutaneous injection. In other animal species blood pressure response was more erratic and unpredicable (91).

The intravenous injection of 1–2 ng/kg caerulein caused in the dog an increase in the blood flow through the duodenal-pancreatic artery. This was concomitant with, and possibly resulting from, the stimulation of pancreatic secretion (92).

(*j*) Bradykinin-like whealing reaction on the human forearm skin for doses above 20–50 ng given by intradermal injection (91).

On all the preparations hitherto tested, caerulein was several times more potent than cholecystokinin-pancreozymin, even on a molar basis. It was even more potent than gastrin, except for the action of this hormone on the gastric secretion in some animal species. In the dog, for instance, low doses of caerulein were highly effective in producing significant amounts of secretion, but large doses were not capable of causing high rates of acid secretion comparable to those seen with maximal doses of gastrin (7).

More than 50 caerulein-like polypeptides have been synthetised in order to contribute to the elucidation of the problem of structure activity relationship for caerulein and cholecystokinin-pancreozymin and in an attempt to dissociate the different actions of caerulein (93).

Without entering into details, it was found that the whole activity spectrum of caerulein depended on the C-terminal heptapeptide, a necessary prerequisite for the activity of this hepatapeptide being the presence at its N-terminus of a O-sulfated tyrosyl residue or another appropriate negatively charged residue. Desulfation of the tyrosyl residue always provoked a drastic reduction of activity, and a similar decay of activity was caused by a shift of the tyrosyl residue towards the C-terminus, as in the gastrins.

Caerulein has so far been employed, with excellent or encouraging results, in cholecystography, in small bowel contrast studies, and in intestinal syndromes characterized by insufficient tone and motility of the gut. It is evident that the polypeptide deserves clinical trial also in digestive disorders attributable to defect of pancreatic and biliary secretion and perhaps in pancreatic scintiscanning. Like pentagastrin, caerulein could be profitably administered in man by nasal insufflation, at dose levels similar to those active by intramuscular injection (94).

Alytesin and alytesin-like polypeptides.—Three polypeptides belonging to this family have been recently isolated in a pure form and submitted to pharmacological screening by two groups of research workers, independently. The formulae below show that we have to do with a new class of polypeptides.

(I) Pyr–Gly–Arg–Leu–Gly–Thr–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
(II) Pyr–Gln–Arg–Leu–Gly–Asn–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
(III) Pyr–Val–Pro–Gln–Trp–Ala–Val–Gly–His–Phe–Met–NH_2

Alytesin (I) is a tetradecapeptide obtained from methanol extracts of the skin of the European discoglossid frog *Alytes obstetricans;* bombesin (II) is again a tetradecapeptide obtained from extracts of the skin of the two European discoglossid frogs *Bombina bombina* and *Bombina variegata variegata* (95); finally, ranatensin (III) is an endecapeptide prepared from extracts of the skin of the American frog *Rana pipiens* (96).

The alytesin-like activity of crude extracts of *Alytes* corresponded to 600–3000 μg of alytesin per g fresh tissue, but it is not certain as yet whether all this activity may be ascribed to alytesin. Similar quantitative data were obtained for crude extracts of *Bombina variegata variegata.* Bombesin or a bombesin-like peptide occurs also in extracts of the skin of *Bombina variegata pachypus,* but so far the polypeptide has not been prepared in a pure form.

Bombesin and alytesin could be easily demonstrated on paperchromatograms and electropherograms of crude or, much better, semi-purified extracts by means of color reactions: Pauly reaction (histidine), coupling reaction with the NNCD reagent or p-dimethylaminobenzaldehyde reaction (tryptophan), Sachaguki reaction (arginine). Alytesin and bombesin were accompanied by other compounds giving similar color reactions.

The spectrum of biological activity of alytesin and bombesin is characteristic, and the distinction of this peptide family from other families is quite easy by means of parallel bioassay. The pharmacological study of bombesin and alytesin is still in progress in several directions. So far the following effects may be considered as well established for alytesin (97):

(*a*) hypertensive action in the dog presenting marked tachyphylaxis. The threshold dose was certainly less than 1 μg/kg by rapid intravenous injection. By intravenous infusion (1 μg/kg/min) the effect declined in spite of continuing the administration of the drug. The effect on blood pressure remained unchanged following pretreatment of the dog with α-adrenergic blocking agents. No cross-tachyphylaxis was observed with Val^5-angiotensin. In the intensity of the hypertensive response alytesin was approximately 10 times less potent than Val^5-angiotensin but the effect of alytesin lasted considerably longer.

(*b*) potent stimulant action on the estrous uterus of the rat. As a rule, alytesin was 2–3 times more potent than bradykinin and at least as potent as synthetic oxytocin. The threshold dose was of the order of 0.01 ng/ml

nutrient liquid and there was a fair dose response relationship. With large doses of alytesin the tonus increase often persisted for hours, in spite of repeated washing of the organ with fresh nutrient liquid. This effect was atropine-resistant.

(*c*) potent stimulant action on the rat and the guinea-pig colon, as well as on the cat ileum. Tachyphylaxis was absent or moderate, and there was again a satisfactory dose response relationship, especially for the guinea-pig colon and the cat ileum. Threshold doses ranged from 0.03 to 0.3 ng/ml nutrient liquid. The effects were again atropine-resistant. In contrast to the colon, the ileum of the guinea-pig was on the whole poorly sensitive to alytesin, which produced repeated spikes of contraction, sometimes lasting for hours. Tachyphylaxis was evident. Atropine inhibited, at least to a great extent, the response to alytesin.

(*d*) remarkable stimulant action on the gastric secretion of the chicken and the dog. In the chicken the intravenous infusion of 10–25 ng/kg/min of alytesin produced an increase in flow of gastric juice of 50–150% and in acid and pepsin outputs. The concentration of pepsin in gastric juice was nearly doubled. Alytesin possessed approximately 5–10% of the potency of caerulein. In dogs provided with denervated gastric pouches, alytesin displayed again about 5% of the effect of caerulein. But in the dog and the chicken the secretagogue action of alytesin was inhibited by atropine.

(*e*) moderate hyperglycemic effect in rats and dogs. In the anaesthetized rat 15 μg/kg of alytesin injected subcutaneously produced a 70% increase of the blood sugar level lasting two hours; 50 μg/kg of the polypeptide produced an increase of glycemia (90%) lasting for more than 5 hours.

In the dog the intravenous infusion of 1 μg/kg/min of alytesin, for a period of 10 min, caused, in addition to hypertension, an increase in blood sugar level, a progressive increase of immunoreactive insulin levels in femoral artery blood (up to 3-fold), finally a moderate reduction of calcaemia.

A number of isolated smooth muscle preparations (rabbit, cat, guinea-pig, and hamster uteri; rabbit, hamster, chicken, and frog intestinal strips; guinea-pig tracheal chain) were insensitive or poorly sensitive to alytesin or showed marked tachyphylaxis. The guinea-pig gall bladder *in situ* was contracted only by intravenous doses of alytesin as high as 50–500 ng/kg.

Bombesin presented a spectrum of biological activity indistinguishable from that of alytesin. On some preparations it appeared slightly less active.

Several fragments of the alytesin molecule have been prepared by synthesis or by enzymic hydrolysis and are now being studied. It has been demonstrated that the C-terminal heptapeptide or, more likely, the C-terminal octapeptide, is necessary for the appearance of activity, and that the synthetic fragments may present pronounced differences in their activity on the various target organs.

Ranatensin showed a spectrum of biological activity similar to that of alytesin on the dog blood pressure and on the guinea-pig ileum and rat

duodenum. In addition it lowered blood pressure in rats and monkeys and contracted the rabbit aortic strip but had no effect on rat aortic strip (98).

Miscellaneous polypeptides.—In addition to the previously described polypeptides which must be considered as firmly established chemical and pharmacological entities, several other active peptides have been traced in the skin of different amphibian species.

Some of them, as already stated, mimic bradykinin in their pharmacological effects, others physalaemin, still others alytesin. However, other peptides cannot be included in any of the preceding groups because of their peculiar spectrum of activity.

The amino acid composition and sequence of some of the "miscellaneous" polypeptides are near to being elucidated; for others a major obstacle to their isolation and study is the scarcity of material; for still others serious methodological difficulties have been met, in part due to the apparent lability of their molecules.

Discussion

It is obvious that the screening procedure used in our research, although covering a progressively increasing number of pharmacological effects, is inadequate to catch all the possible actions possessed by the polypeptides of the amphibian skin. Consequently, it is very possible that the activity spectrum of the known polypeptides is actually broader than that so far established, and it is similarly possible that other peptides completely escape our attention because their activity lies beyond the limits of our screening methods.

At any rate, the study of the active peptides in amphibian skin has been an inexhaustible source of surprise. First physalaemin was found, similar to eledoisin of the posterior salivary glands of an octopod and to substance P of the mammalian intestine and brain; then authentic bradykinin, identical to that found in mammalian plasma, and bradykinin-like peptides; then caerulein with its astonishing similarity to the gastro-duodenal hormones of mammals; finally alytesin and bombesin with their peculiar spectrum of activity. Recent data point to the occurrence of amphibian peptides displaying effects similar to those produced by other mammalian hormonal peptides.

What is the significance of this constellation of tremendously active peptides in amphibian skin? Why does the genetic code cause the allineation in the same sequence of the same amino acid residues in amphibian skin, in the posterior salivary glands of octopods and in different, sometimes highly differentiated tissues of mammals? Finally, what is the function of active peptides in amphibian skin?

These questions must be left open until the analytic, descriptive phase of research is accomplished or at least more advanced.

Concerning the possible function of the polypeptides in amphibian skin, one could tentatively suggest that they may interfere in some basic function

of the skin, for example in the regulation of the secretion of the skin or in the control of water and electrolyte exchanges through the skin. However, it should be kept in mind that often polypeptides seem to be mainly localized in the cutaneous glands, the secretion of which is believed to be only external.

It is obvious that the same function may be displayed in the different amphibian species by different polypeptides, and it is conceivable that polypeptides inactive in our screening systems are active on amphibian skin.

And now a final consideration. We are certain that the study of the spectrum of active peptides and of biogenic amines occurring in the skin of the largest possible number of amphibian species will be of interest not only from the viewpoint of comparative biochemistry and pharmacology but also from that of biochemical taxonomy.

Results already obtained with leptodactylid frogs and bufonids confirm the validity of this assumption, proving that extensive research in the field of natural compounds has a significance that largely transcends the limits of a single branch of biology.

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