12. Biogenic Amines

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Introduction

Very strong biochemical and metabolic activity occurs in the batrachian skin, where many different substances are produced. There are many important physiological and ecological functions related to the skin, such as passage of water and electrolytes, essential respiratory activity, thermoregulation, secretion of defensive venoms, mimicry, and others. The complicated structure of the secretive layer of the integument emphasizes its intensive cellular activity, especially in such topographically specialized areas of the body as the parotoid, inguinal, lateral, or tibial glands.

During the past ten years, about four hundred amphibian species have been collected throughout the world, and methanol or acetone extracts of the dried or fresh skins of these creatures have been subjected to chemical and biological screening. From this extensive investigation emerged the generalization that the amphibian skin is a formidable storehouse of aromatic biogenic amines (with their precursors and metabolites) and of polypeptides active on smooth muscle and on external secretions. Several new amines and polypeptides have been identified and their structures elucidated. Amines and polypeptides are, of course, only a small part of the biogenic molecules occurring in the amphibian skin. Our screening was limited to amines and polypeptides because of their considerable pharmacological interest, on the one hand, and because of the research aims of our working group, on the other.

While analytic data were accumulating, it soon appeared that amine and polypeptide spectra varied conspicuously among the different genera, species, and perhaps subspecies as well. To be specific, it ap-

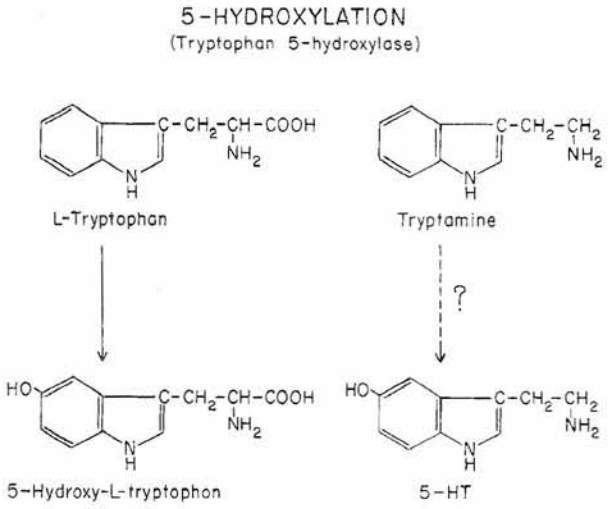

Fig. 12–1. Enzymatic events that occur in the toad skin: 5-HYDROXYLATION (Tryptophan 5-hydroxylase).

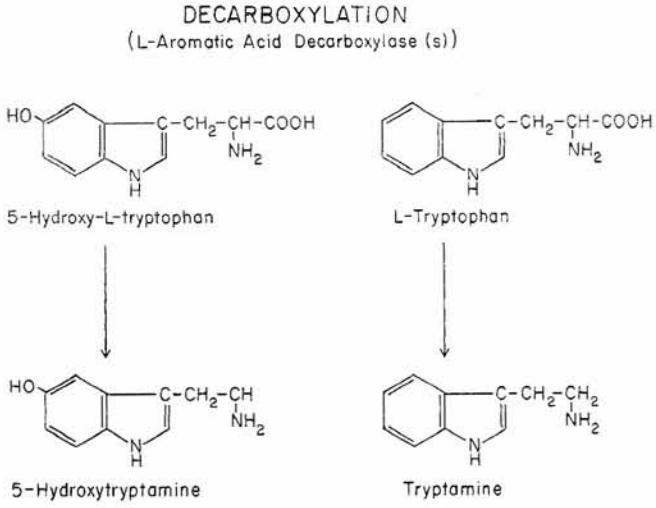

Fig. 12–2. Enzymatic events that occur in the toad skin: DECARBOXYLATION (L-aromatic acid decarboxylase[s]).

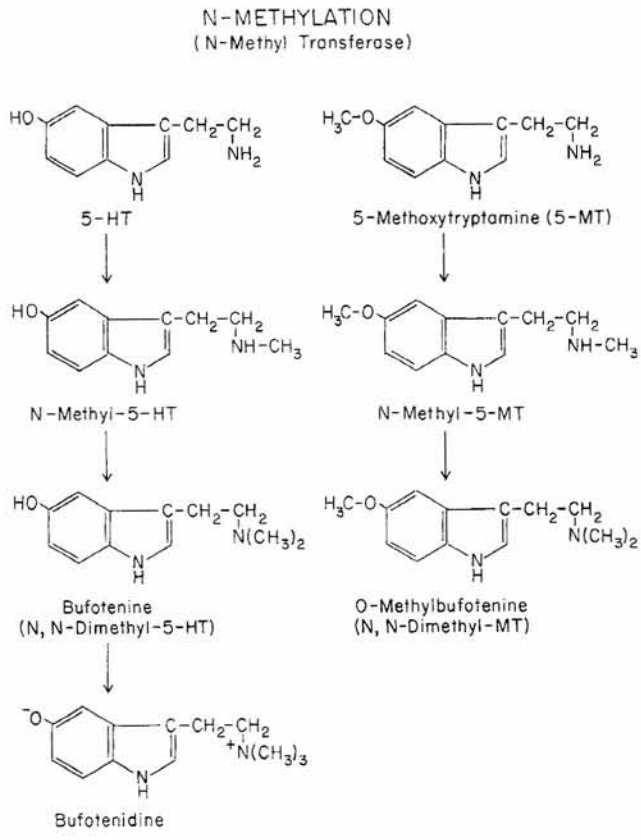

Fig. 12–3. Enzymatic events that occur in the toad skin: N-METHYLATION (N-methyl transferase).

peared that the spectrum of biogenic amines and polypeptides could be profitably used, at least as a subsidiary tool, in biochemical taxonomy and for assessing evolutionary relationships. Among the amphibian species and subspecies considered in this extensive study, sixty-five belong to the genus *Bufo*. Three species come from Europe, nine from Africa, five from Asia, and the others from the Americas.

The skin of these *Bufo* species provides a spectacular representation of indolealkylamines. There is no animal or vegetable tissue known that can compete with the toad skin in variety and quantity of indolealkylamines and of their precursors and metabolites. Approximately twenty indole derivatives have been identified so far in extracts of toad skin, and this number will grow.

It is obvious that the occurrence of a given indole derivative implies the occurrence of the enzyme systems catalyzing its biosynthesis. In the case of toad indolealkylamines at least the following enzyme systems are required for their biosynthesis and metabolism: *tryptophan-5-hydroxylase,* the enzyme responsible for the 5-hydroxylation of tryptophan, that is, responsible for the biosynthesis of 5-hydroxytryptophan, the obligatory precursor of all 5-hydroxy- and 5-methoxyindolealkylamines; *aromatic L-amino acid decarboxylase*(s), capable of decarboxylating tryptophan to tryptamine and 5-hydroxytryptophan to 5-HT; *N-methyl transferase,* responsible for the transformation of the primary into the secondary and tertiary amine, and then into the quaternary ammonium base; *5-hydroxyindole-O-methyl transferase,* which causes the methylation of the hydroxy group

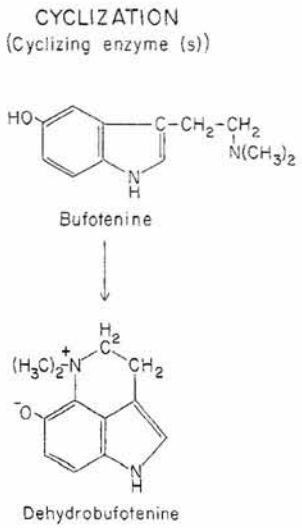

Fig. 12–4. Enzymatic events that occur in the toad skin: CYCLIZATION (cyclizing enzyme[s]).

of the nucleus, forming 5-methoxyindoles; *cyclizing enzymes*, involved in the biosynthesis of dehydrobufotenine, proceeding by dehydrogenation of bufotenine to a *para*-quinonid structure, which then cyclizes by nucleophilic addition of the $-N(CH_3)_2$ group (Robinson et al. 1961); one or two, or even more, *conjugases*, capable of linking sulphuric acid to the phenolic hydroxy group (*O-sulphoconjugase* or *5-sulphoconjugase*) on the side, and of linking again sulphuric acid or other acids to the pyrolic $>NH$ group, in position 1, on the other side (*1-conjugase[s]*); and finally *monoamineoxidase*, responsible for the oxidative deamination of primary and secondary indolealkylamines. Figures 12-1 to 12-8 give a synopsis of the enzymatic events that occur or are likely to occur in the toad skin.

It is probable that several of these enzyme systems are highly specific. But enzymes are proteins, that is, precise chemical structures manufactured in the cell from regular subunits by a complex coding process. Keeping this concept in mind, it is obvious that each amine and each precursor or metabolite may be considered a trait by which we can trace species evolution, and, at the same time, we can get information about the relationships existing among closely related species. Thus, our data might contribute to biochemical taxonomy, which, in its broadest sense, has possibly even greater validity than traditional taxonomy based on morphological characters, such as somatic or osteological structures, size, or coloration.

However, it must be stressed that present data on the spectra of biogenic indolealkylamines should be considered only preliminary to a more extensive investigation and that all inferences drawn from these data should be accepted with prudence. For example, considerable hindrance to the theoretical elaboration of our analytical data is the conspicuous quantitative differences in the amine content found in different individuals of the same species, even when collected at the same time and at the same place. These differences may sometimes even simulate changes in the amine spectrum, due to the occasional absence or presence of a given amine. It is obvious that this physiological handicap may be largely overcome by screening pools of numerous specimens or by numerous individual screenings, which has been done, in our study, whenever possible. Our present data are also unable to demonstrate any kind of reliable sex differences.

In some previous works (Capurro and Silva 1959; Hunsaker, Alston, Blair, and Turner 1961; Wittliff 1962, 1964; Porter 1962, 1964, 1966; Porter and Porter 1967) paper chromatographic analysis of the parotoid-gland secretion of toads has been used in assessing intragroup or intergroup relationships. Research reported in the present chapter, together with that in preceding papers (Cei and Erspamer 1966; Cei, Erspamer, and Roseghini 1967, 1968) and other papers in preparation, represent an effort to check the actual value of this kind of biochemical investigation in as many amphibian species as possible.

The species considered in this chapter, together with data concerning number and state of the skins and date and place of collection, are indicated in Appendix I. Acetone was the solvent routinely used for extracting the skins. In fact, 80 percent acetone is the most suitable solvent for a complete recovery of tissue indolealkylamines. For details on the simple extraction procedure and for a description of paper chromatographic analysis and biological methods, reference has been made to other papers (Erspamer, Roseghini, and Cei 1964; Erspamer, Vitali, Roseghini, and Cei 1964, 1967). The problem of whether the amine spectrum found in dried skins may be considered qualitatively and quantitatively comparable with that occurring in fresh skins has been discussed and will be discussed elsewhere (Cei, Erspamer, and Roseghini 1967).

A number of indole compounds found in the toad skin have been identified in the present report with O-conjugates and 1-conjugates of amines with sul-

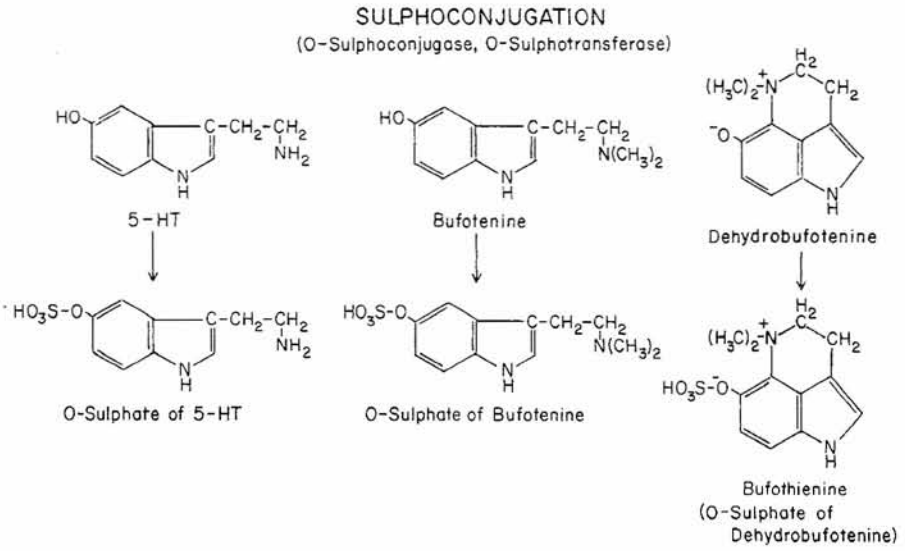

Fig. 12–5. Enzymatic events that occur in the toad skin: O-SULPHOCONJUGATION (O-sulphoconjugase, O-sulphotransferase).

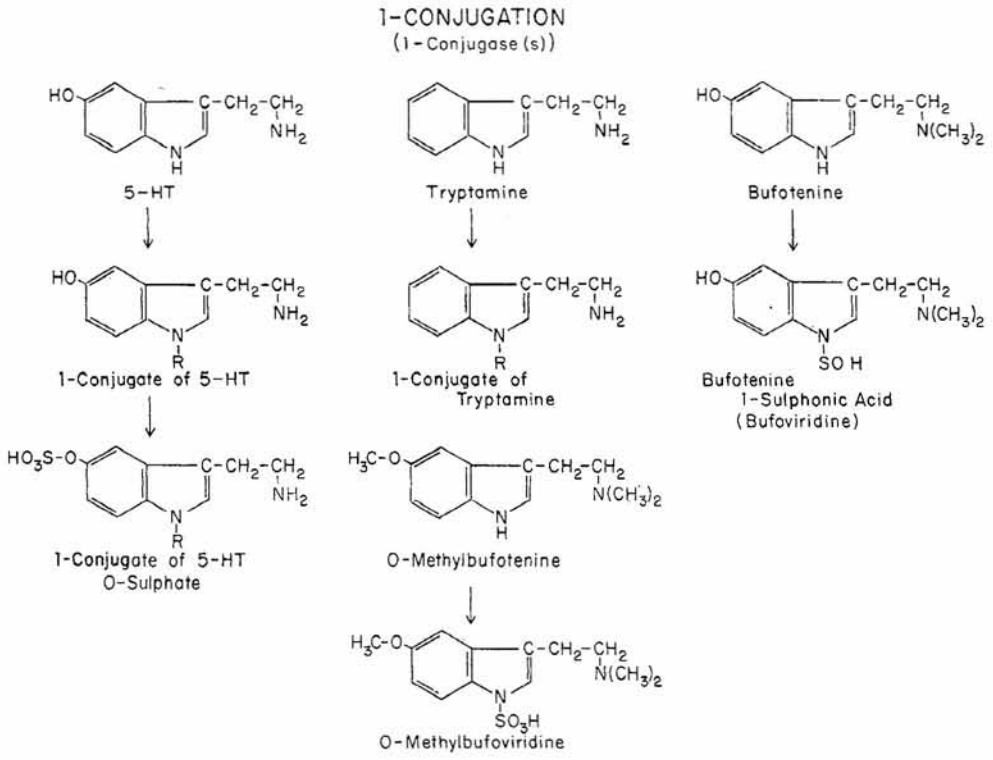

Fig. 12–6. Enzymatic events that occur in the toad skin: 1-CONJUGATION (1-conjugase[s]).

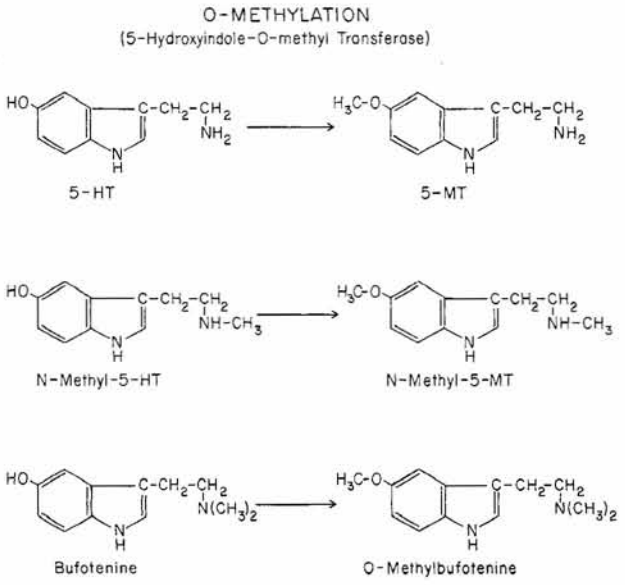

Fig. 12–7. Enzymatic events that occur in the toad skin: O-METHYLATION (5-hydroxyindole-O-methyl transferase).

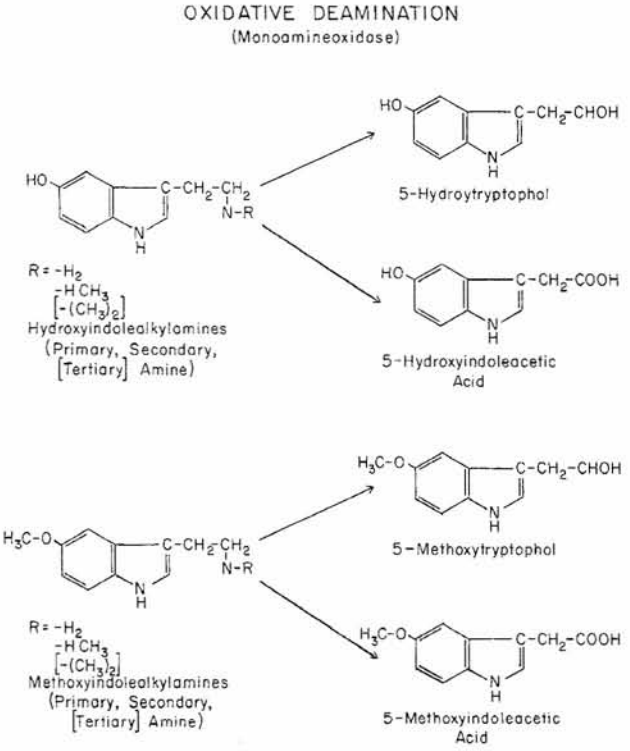

Fig. 12–8. Enzymatic events that occur in the toad skin: OXIDATIVE DEAMINATION (Monoamineoxidase).

phuric acid or other acids. It should be stated that this identification is only tentative. In fact, in spite of considerable positive evidence, only the synthesis of the conjugated compounds shown in the diagrams will prove or disprove their identity with the natural compounds found in the toad skin. This reservation does not apply to bufothionine, whose identification with the O-sulphate of dehydrobufotenine is certain. Prudence is more necessary with bufoviridine, which until now was considered bufotenine O-sulphate; it is possibly bufotenine 1-sulphonic acid.

Taxonomic Application of the Biochemical Data

South American Toads

We will begin by discussing the neotropical stocks of the South American continent, whose ancestral relationships and tentative evolutionary history have been analyzed in another chapter (Chap. 6). These involve early Tertiary genocenters, both in Guiana-Brazilian and Patagonian shields. Two different, perhaps opposing, or diverging stocks are evident on the basis of geographic distribution and morphological arguments, besides the more recent tests of genetic compatibility. One is a western, or Andean stock, which can be provisionally designated as a "southwestern" section of the "thin-skulled line," corresponding to the *spinulosus* complex. Second, there are stocks in South America, probably related to the "southern line" of *valliceps*-like features, such as the *marinus* and *granulosus* groups, which are widely distributed on the entire continental mass, eastward from the Cordilleran range, which is the realm of the *spinulosus* stock.

We have examined most of the taxonomic units of the *spinulosus* complex. In spite of the general similarities within this group, advanced speciation can be recognized in many of its forms, and the ecological and paleogeographic features of its radiation have been discussed by one of us in another chapter (see Chap. 6). The screening of indolealkylamines, which imply specific enzymatic activities, presents a common physiognomy of all the taxonomic units. All species of the complex present a lively biosynthesis of indolealkylamines. However, 5-HT, together with N-methyl-5-HT, is generally absent in the skin, owing to the intensity of N-methylating processes, followed by cyclization and conjugation processes.

From this common pattern some distinctive fea-

tures emerge for some of the species of the *spinulosus* complex. *B. flavolineatus,* for example, is characterized by an unusually intense and complete N-methylating activity leading to the formation of large amounts of not only bufotenine but also bufotenidine, and by a relatively reduced cyclizing activity, with the consequence of reduced amounts of dehydrobufotenine and bufothionine. Something similar may be observed for *B. trifolium.*

B. chilensis, in turn, is characterized not only by the regular occurrence in its skin of the usual O-sulphate of dehydrobufotenine (bufothionine), but also by the occurrence of the O-sulphate of bufotenine, a derivative found so far only in the *calamita* group from Europe and in some groups of the western section of the American "northern line," but completely lacking in all the representatives of the "southern line." The O-sulphate of bufotenine has also been traced in the "*B. spinulosus* population" of Cajamarca and exceptionally (in one skin out of ten) in the *B. spinulosus* population of San Isidro, Mendoza. Additional research on more specimens is necessary before making definitive assumptions about different characteristics in the amine spectrum of *B. atacamensis* and *B. limensis.*

However, an independent position is demonstrated by our screening for the small *B. variegatus* from the cold forest of Valdivia. It is surely a very ancient Patagonian form, probably from some early Tertiary austral stock of primitive toads, but its amine spectrum differentiates it from any other form of the southwestern section. Its high content of bufotenine is peculiar, and the occurrence of dehydrobufotenine and bufothionine is negligible.

An ancient neotropical branch of the "southern line" has been suggested on the basis of morphological, osteological, and embryological arguments (genetic compatibility). Undoubtedly it is impossible to characterize, with our biochemical evidence, the primeval steps of its phyletic radiation, because of the intervention of the peculiar pattern of speciation, the environmental challenges, and the probable effects of convergent evolution. However, at the level of the present speciation and in accordance with the relatively limited boundaries of the "groups," there is a remarkable agreement between the phenotypical metabolic expression, which the biochemical evidence reflects, and taxonomic status.

For example, *B. marinus* shows a clear uniformity in the amine spectra for all the populations, from Mexico to Surinam, in the Amazonian environment. But the smaller, high Amazonian *B. poeppigi* shows as a "good specific character" a lowered activity of the enzyme system that leads to the formation of dehydrobufotenine. Likewise, a similarity is found in the spectra of *B. marinus* and *B. ictericus,* thus strengthening the evidence that these two, now geographically disjunctive, forms, from the Guiana-Amazonion range and the eastern forest coastal range, are related. They were surely closely related prior to the geological events leading to the dryness of the present central area of Chaco and caatingas. Considerable similarity of the amine spectra indicates a probable late common branching of *B. paracnemis* and *B. arenarum,* perhaps in agreement with some recent paleontological findings (Casamiquela, in press).

The so-called *granulosus* group is a much discussed taxonomic complex. *B. granulosus* Spix has been fractionated into a number of subspecific units, whose geographic distributions, still poorly known, cover much of the continental area, from the Panamian lowlands to the pampean southern range. For morphological, physiological, and probably genetic reasons, many of these so-called morphological subspecies may actually be good biological species, and in a number of cases their evident overlapping can support such a logical assumption. At any rate it is possible that – as in other neotropical forms – the speciation and differentiation of some of these populational groups could have occurred in a relatively short space of geological time. For example, the geographic distribution of *B. major* resembles the geographic xeric central range of the giant toad *B. paracnemis,* which extends from the San Francisco valley to the Argentine Chacoan woods. Screening of the amine spectra of the presently available species demonstrates that the enzyme activity is fundamentally the same in *B. granulosus goeldi,* in *B. major,* in *B. fernandezae,* and in *B. pygmaeus.* Only some quantitative differences can be traced. For example, dehydrobufotenine is always less abundant in the Chacoan toad *B. major* than in other examined forms of the complex. The spectra of indolealkylamines are strikingly similar for the *granulosus* stock and *B. cruci fer* stock, which may be a very primitive toad (Chap. 18).

On morphological grounds, a strong relationship has also been suggested between the *granulosus* group and the Caribbean forms of the *peltacephalus* group. The cranial morphology and other arguments have been discussed. However, if one compares the amine spectrum of the skin of *B. peltacephalus* from

Cuba, a striking difference can be noted, indolealkylamines being represented in the *B. peltacephalus* skin only by small amounts of 5-HT and trace amounts of dehydrobufotenine. If some relationship between the insular radiation and an ancestral common neotropical stock is assumed, geographic isolation may have played a very important role in the biochemical evolution. The reduction or shortage of some enzymic activities could thus depend on either some genetic selective factor or genetic isolation (genetic drift?). Similar events – under different environmental factors – have perhaps occurred in the case of *B. atacamensis*, undoubtedly a derivative form from a primitive *chilensis* stock, whose reduced amine spectrum might be strictly conditioned by its complete isolation along the few rivers of the azoic Atacama Desert.

Central and North American Toads

The evolutionary radiation of American toads is enormous, but in the last years some tentative arrangements of their fundamental main lines have been suggested. Tihen (1962) has used osteological arguments, and Blair (1963, 1964, 1966), has pointed out genetic compatibility in interspecific crosses. Two primeval main lines have been tentatively suggested by Blair for the North American toads. They have been named "northern line" (or "thin-skulled line") and "southern line" (or "broad-skulled line").

The most representative elements of the northern line, more precisely of its western section, are the toads of the *boreas* group and related groups (Chap. 7). The amine spectrum in the skin of the *boreas* toads is rather homogeneous, which indicates a close taxonomic affinity among the species of this group, but it shows no peculiar characteristics. The spectrum is very similar to that presented by the European *B. bufo bufo*. *B. canorus*, however, an isolated form from the high summits of Sierra Nevada, California, differs from *B. boreas* in the very high activity of N-methyl transferase, cyclizing enzymes, and O-sulphoconjugase, the last-named leading to an unusual accumulation of bufothionine.

Although they belong to the same western section of the northern line, the other groups of this section present one of the most characteristic spectra of indolealkylamines that can be encountered in toads, which sometimes permits an immediate diagnosis of species. All toads belonging to the *punctatus*, *debilis*, *marmoreus*, and *alvarius* groups show high activity in the indolealkylamine biosynthesis and a pronounced activity of N-methylating enzymes. Cyclizing enzymes are highly active in the *punctatus* and *debilis* groups, while absent or extremely inactive in the *marmoreus* and *alvarius* groups. But the most striking biochemical feature of these groups of toads is the occurrence in their skin of very vigorous conjugation processes. Conjugation may be observed not only for dehydrobufotenine, where this is present, but, what is far more characteristic, also for bufotenine and eventually for 5-HT. In the *punctatus* and *debilis* groups only the 1-conjugate of bufotenine (bufoviridine or bufotenine 1-sulphonic acid) is apparently present. (Trace amounts of bufotenine O-sulphate may have escaped our attention.) In the *marmoreus* and *alvarius* groups, both bufoviridine and, in considerably larger amounts, the O- or 5-sulphate of bufotenine are present. It may be seen that in its indolealkylamine spectrum the *marmoreus* group is indistinguishable from the European *calamita* group, whose early relationship with some primeval representatives of the northern line has been discussed (Blair 1963).

The apparently peculiar behavior of *B. kelloggi*, with its enormous accumulation of dehydrobufotenine and lack of conjugated amines, cannot be considered seriously, because only one specimen was available for the screening. On the contrary, the exceptional position of *Bufo alvarius* may be emphasized on the basis of a careful study. The skin of *Bufo alvarius*, the only one among the skins of the sixty-five toads examined, uniquely possesses a formidable O-methyl transferase activity, which leads to the accumulation of enormous amounts of N, N-dimethyl-5-methoxytryptamine (O-methylbufotenine), and small amounts of N-methyl-5-methoxytryptamine. O-methylbufotenine constitutes sometimes as much as 15 percent of the dry weight of the parotoid and tibial glands of *B. alvarius*. The methoxytryptamines may give origin, following attack by monoamine-oxidase, to the pertinent deaminated metabolites 5-methoxytryptophol and 5-methoxyindole acetic acid, which can regularly be found in the extracts of the *B. alvarius* skin.

The *americanus* group is a well-known set of eastern and in some cases (*B. hemiophrys*, *B. microscaphus*) western species, whose evolutionary trends and probable phyletic history have been thoroughly discussed by Blair (1963). It is a very distinctive group in spite of its morphological and biological radiation, which is probably connected with the late Pliocene and Pleistocene events. The indolealkyla-

mine spectra accord well with the general relationship of the group and its intrageneric differentiation. A reduced cyclizing activity is the rule, resulting in lack or extreme scarcity of dehydrobufotenine. The only exception is the isolated western populations of *B. hemiophrys*. Conjugases of any kind are completely lacking. In addition, specific quantitative enzymatic trends are evident for all the examined forms: *B. americanus, B. terrestris, B. woodhousei, B. microscaphus,* or *B. hemiophrys,* not to mention the intraspecific and geographical variation.

The small eastern toad *B. quercicus* from the forested southeastern United States is perhaps a derivative from the eastern stock. Only moderate amounts of 5-HT and of dehydrobufotenine can be found in its skin, indicating reduced activity of the pertinent enzymes. Blair states in his discussion that *B. quercicus* appears at first glance "a miniature of *B. woodhousei.*" Due to the strong agreement existing between the amine spectra and the general arrangement of the *americanus* group as a "low crested" representative of the "northern line," other evidence could be added about the postulated origin of *B. quercicus* from the *americanus* group and about the allocation of this diminutive species of the "northern line."

The "southern line," as treated in the tentative arrangement by Blair, and considering the previous osteological considerations of Tihen (1962), is primarily represented by the *valliceps* stock and its allies. *Bufo valliceps,* of the southern states and Mexico, is closely related to a number of Mexican and Guatemalan forms previously discussed under their specific status by Porter (1964). Our data pertain only to *Bufo valliceps,* in whose skin the only representative of idolealkylamines is 5-HT, indicating the absence of methylating, cyclizing, and conjugating enzymes. (Compare App. I, Table 3.) A great evolutionary radiation seems to have occurred in the heavy-skulled toads of the "southern line," both in Central and in South America. As discussed in other chapters of this book, the primeval relationships of the most remote branches of the American bufonid lines are still unclear, and paleogeographic problems are also related to the more ancient radiating steps of all these groups. Different kinds of evidence may support some remote relationships between *valliceps* and the neotropical stocks, such as the *marinus, granulosus,* and *typhonius* groups. But, on more objective grounds, we must point out that if the biochemical methods and criteria – in our case the amine spectrum – can be very useful for a better screening of the interspecific and intraspecific relationships, this support could only be of relative utility in some special or general cases of phylogenetic reconstruction.

In spite of the above-mentioned reservations in the use of biochemical affinities as a mean of phyletic comparison, we find that the other evidence of taxonomic affinity between *B. cognatus* and *B. speciosus* is also strengthened by the amine spectra of the two species. This is a very elegant example of total agreement between biochemical and morphological characters, and the genetical value of metabolic pathways of indolealkylamines as phenotypical expression of the genetic code is here strikingly focussed. (The *cognatus*-group toads are specialized for a life in desert environments and range over the very extensive Sonoran area, from Texas to Mexico.)

Sulphoconjugating enzymes are lacking in all presently studied Central American toads (*B. luetkeni, B. coccifer,* and *B. coniferus*) except in *B. canaliferus,* from Mexico to Guatemala, in which this enzymic set is present and very active. True evolutionary trends within genetically defined groups can thus be ascertained, in terms of specific genetic information. The information is to some extent independent of the environmental effects, but is subject, however, to general geographic variation.

The *guttatus* and *typhonius* groups, probably of ancient neotropical origin and most likely only recent immigrants to Central America, are still poorly known. It is also difficult to compare their peculiar amine spectra to the previously discussed spectra of the related forms of the *valliceps* group. Considerable activity of cyclizing enzymes and of o-sulfoconjugase may be reported for the *typhonius* toads from Panama. Similarly, a formidable activity of N-methylating and cyclizing enzymes, leading to the accumulation of enormous amounts of dehydrobufotenine (up to 18,000 μg/g dry skin), has been ascertained in Costa Rican specimens of *B. haematiticus.* A very independent ancient radiation of many of these forms can thus be postulated from comparative biochemical evidence.

Eurasian Toads

Eurasian stocks must be divided into three distinct groups.

Toads belonging to the Indonesian or Malayan group (*B. asper* from Malaya and *B. juxtasper* from Borneo) are virtually lacking indolealkylamines; the traces that are present indicate extremely torpid

enzyme activity. The same picture may be encountered in the sympatric, related bufonid genus *Pseudobufo subasper* from Malaya.

On the other hand, toads belonging to the continental Euro-Indian group, although including species from very different localities (*B. melanostictus, B. bufo, B. stomaticus*), present a rather uniform amine spectrum, which indicates a lively activity of all enzyme systems involved in the biosynthesis of indolealkylamines, with the exception of 1-conjugase.

Finally, toads belonging to the third group (*B calamita* and *B. viridis*), with European and Mediterranean ranges, are sharply characterized by absence or extreme scarcity of cyclizing enzymes (hence absence of dehydrobufotenine and bufothionine) accompanied by very lively N-methylating and conjugating (both 1- and 5-conjugating) activities, leading to the accumulation of large amounts of bufoviridine and, to a lesser extent, of the O-sulphate of bufotenine. It should be stressed here that the same conjugated derivatives may be found in some American toads of the western section of the so-called northern line.

African Toads

There is remarkable uniformity among all the hitherto examined African toads (*B. mauritanicus, B. regularis, B. kisoloensis, B. berghei, B. funereus, B. garmani, B. rangeri, B. maculatus, B. carens*), despite the important diversity of their chromosomal constitution, which, as reported by Bogart (1968), is 2n = 20 for *B. regularis, B. gutturalis, B. garmani, B. rangeri, B. brauni, B. latifrons,* and 2n = 22 for *B. mauritanicus, B. gariepensis, B. rosei,* and *B. carens.* We are dealing with a general paleotropic stock, whose species are perfectly capable (with the exception of *B. carens*) of synthesizing 5-HT but are completely lacking N-methyl transferase activity and hence cyclizing activity. Conjugases, on the contrary, both 5 (O)-conjugase and 1-conjugase, are highly active in all species. African toads are further characterized, again with the exception of *B. carens,* by the content of other indole derivatives (emerald green spot, see Appendix I, Table 1) and by the content of a peculiar hydroxyphenylalkylamine, *kisoloensin,* the study of which is in progress. Among the examined African toads, *B. carens* occupies a unique position, since it contains large amounts of a 1-conjugated derivative of tryptamine, which is a quite unusual indolealkylamine in the amphibian skin.

Needless to say, screening of other species is necessary before drawing acceptable conclusions on the evolutionary relationships existing between the different species of the African stock. For the present we may hold that our biochemical data do not disagree with Bogart's assumption (Chap. 10) that all the African toads possessing 20 chromosomes may likely be derived from a common ancestor having 22 chromosomes.

Conclusion

The amine and polypeptide spectra of the amphibian skin vary among the representatives of different families, but also among different genera, species, and subspecies. The specific spectrum of biogenic amines and polypeptides can be profitably used in biochemical taxonomy and for assessing evolutionary relationships, as in our present screening of sixty-five forms belonging to the genus *Bufo.* The skin of the examined *Bufo* offers a spectacular array of indolealkylamines; approximately twenty indole derivatives have been identified so far. The occurrence of a given indole derivative necessarily implies the occurrence of the enzyme systems catalyzing its biosynthesis; it is probable that the enzyme systems are highly specific, since they are proteins, that is, precise chemical structures manufactured in the cell from regular subunits by a complex coding process. Thus, each amine and each precursor or metabolite may be considered to be a trait by which we can trace species evolution and get information about the relationships existing among more or less closely related species or species group. In this way, and by employing the quantitative differences in the amine content of different individuals of the same species, a number of paleotropical, palearctic, Oriental, nearctic, and neotropical toads have been studied and their specific amine spectra screened and discussed.

It has been mentioned that all the hitherto examined African toads are remarkably uniform, despite their chromosomal diversity. This paleotropical stock is only capable of synthesizing 5-HT; however it is completely lacking N-methyl transferase activity and cyclizing activity, but not conjugase activity (5(O)-conjugase and 1-conjugase). Among the African toads, *B. carens* occupies a unique position, since it contains large amounts of a 1-conjugated derivative of tryptamine, a quite unusual indolealkylamine in the amphibian skin.

The Eurasian stocks can be divided into three distinct groups: a Malayan group virtually lacking

indolealkylamines; a Euro-Indian group (*B. melanostictus, B. bufo, B. stomaticus*), presenting a rather uniform amine spectrum, which indicates a lively activity of all enzyme systems involved in the biosynthesis of indolealkylamines, with the exception of 1-conjugase; a third group, the *calamita* group (including *B. viridis*), sharply characterized by lack or extreme scarcity of cyclizing enzymes, accompanied by very lively N-methylating and conjugating activities, leading to the accumulation of large amounts of bufoviridine and of O-sulphate of bufotenine. The same conjugated derivatives may be found in some American toads of the "northern line" of Blair.

Two primeval main lines of the American toads have been tentatively proposed by Blair; the "northern line" and the "southern line." The amine spectra indicate a close taxonomic affinity among the species of the first section, with the *boreas* group being very similar to the European *B. bufo*. Other representatives of this section, belonging to the *punctatus, debilis, marmoreus,* and *alvarius* groups, show a general liveliness of the indolealkylamine biosynthesis and a pronounced activity of N-methylating enzymes. Cyclizing enzymes are highly active in the *punctatus* and *debilis* groups, while lacking or very torpid in the *marmoreus* and *alvarius* groups. Another striking biochemical feature of these toads is the occurrence of vigorous conjugation processes, producing some specific spectra that, as in the case of *marmoreus* group, are indistinguishable from the European *calamita* group, whose early relationships with some primeval representatives of the "northern line" have been discussed (Blair 1963). The exceptional position of *B. alvarius* from the Arizona desert may also be emphasized, the only one among the sixty-five toads examined having the unique property of a formidable O-methyl transferase activity, which leads to the accumulation of enormous amounts of N,N-dimethyl-5-methoxytryptamine.

The indolealkylamine spectra accord well with the general relationship of the *americanus* group and its intragroup differentiation, which is probably related to late Pliocene and Pleistocene events. The agreement existing between the amine spectra and the general taxonomic arrangement may add evidence to the postulated origin of *B. quercicus* from the *americanus* group.

The "southern line," as tentatively presented by Blair, has been discussed following our present biochemical methods and criteria. The absence of methylating, cyclizing, and conjugating enzymes is the rule in *B. valliceps*, in whose skin only 5-HT is reported. The great taxonomic affinity between *B. cognatus* and *B. speciosus* is supported by their amine spectra. Sulphoconjugating enzymes are lacking in the *cognatus* and *coccifer* groups and in *B. coniferus* and *B. luetkeni* of the *valliceps* group, but they are present in the *canaliferus* group. A very independent ancient radiation of the *guttatus* and *typhonius* groups may be postulated from the comparative biochemical evidence.

There is a western or Andean stock that is provisionally designated as a "southwestern" section of the thin-skulled line, corresponding to the *spinulosus* complex. Screening of the indolealkylamines reveals a common physiognomy for all populations studied, but from this common pattern some distinctive biochemical features emerge. This is the case for *B. flavolineatus* and *B. trifolium* from Peru, *B. chilensis* and *B. atacamensis* from Chile, and *B. variegatus* from the cold Valdivian forest.

Finally, an ancient neotropical branch of the "southern line" may be identifiable from the phenotypical metabolic expression of the enzymatic activity of the skin. For example, the clear uniformity of the amine spectra for all the populations of *B. marinus*, from Mexico to Surinam, has been stressed, as well as the similarity between *B. marinus* and *B. ictericus*, now geographically disjunct forms. The considerable similarity of the amine spectra also indicates a late common branching of *B. paracnemis* and *B. arenarum*. Likewise, the many populations of the poorly understood *granulosus* complex show a common enzyme activity, but quantitative differences of significance may be traced, which are related to the probable speciation in the group. There is a noticeable difference, on the other hand, between the *granulosus* group and the morphologically related forms of the *peltacephalus* group.

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