for mapping functional catecholamine neuronal pathways and sites of neurohormonal action.

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Hormone-Induced Sexual Differentiation of Brain and Behavior in Zebra Finches

Abstract. *The male zebra finch sings, whereas the female does not. This behavioral dimorphism is correlated with the presence of morphological sex differences within the neural substrate that mediates this behavior, the song system. When a female chick is exposed to 17β-estradiol her song system is subsequently masculinized. Either testosterone or 5α-dihydrotestosterone may then induce such a female to sing when an adult.*

Adult vertebrates are sexually dimorphic with respect to various outward signs of brain function, among which are patterns of sexual behavior and of gonadotropin secretion. In mammals and birds, the sexuality of the brain develops under an influence of androgens or estrogens exerted during the perinatal period. This suggests that sex differences in the neural substrates that mediate these brain functions are also determined at this time (*1*). The sites of brain sexual differentiation in mammals have been implicated indirectly by brain lesion, brain stimulation, steroid autoradiography, and local hormone implants (*2*). Sex differences in brain structure have also been documented (*3*). However, since the neural circuits that mediate sexually dimorphic brain functions in mammals have been characterized only roughly, the specific hormone target sites within the developing mammalian brain and the cellular or functional consequences of their sexual differentiation are not well understood.

We have examined hormone influences on the development of song and of the brain nuclei that control this behavior in zebra finches (*Poephila guttata*). Among vertebrates, the avian song system is one of a few examples of discrete neural systems that mediate specific behaviors, and thus is particularly advantageous for neurobiological investigation. The song system consists of a chain of distinct brain nuclei that directly participate in the efferent motor pathway responsible for song (*4*). In the zebra finch, only males sing, and all brain nuclei of the song system are much larger in males than in females (*5*). The studies described in the present report demonstrate that 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT) influence the establishment of these differences in functional capacity and in brain architecture. This is the first such clear-cut example of a direct association between the sexual differentiation of brain and behavior.

The goals of our study were to investigate: first, if androgen or estrogen influences sexual differentiation of the song system (*6*); second, which hormone acts where; third, when the song system is sensitive to hormone; and fourth, if sexual differentiation of the functional capacity for song follows morphological masculinization of the song nuclei. Our

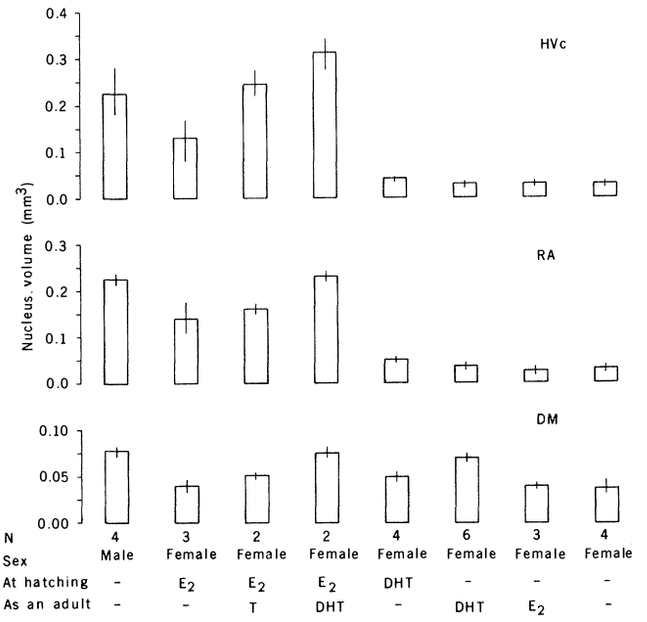

Fig. 1. Effect of various hormone treatments on the volume of HVc, RA, and DM. Mean volumes with ranges are plotted on the ordinate in cubic millimeters (*9*). Below each column is the number (*N*) of birds used and the hormone treatment given to that group; *E_2*, *DHT*, and *T* designate, respectively, 17β-estradiol, 5α-dihydrotestosterone, and testosterone. Bars denote no treatment.

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study focused on three brain nuclei: the nucleus robustus archistriatalis (RA) and the nucleus hyperstriatum ventrale, pars caudale (HVc), both of which lie in the telencephalon (*4*), and dorsomedial intercollicular nucleus (DM), which lies in the brainstem (*7*).

Zebra finch chicks were hatched in an incubator and a Silastic pellet containing steroid was implanted subcutaneously in each one (*8*). The chicks were then transferred to the nests of Bengalese finch foster parents. One group of chicks received 50 μg of E_2, and a second group of chicks received 50 μg of DHT (*9*). Birds which were implanted with hormone at hatching will be referred to as either E_2- or DHT-males or females. Neither hormone treatment affected the development of the song system in the male. Both E_2- and DHT-males possessed song systems whose volume was equivalent to that of normal males with intact gonads, and both groups of hormone implanted males developed a stereotyped song (*10*). This is unlike the influence of hormones on the sexual differentiation of Japanese quail; early exposure to estrogen appears to suppress the emergence of copulatory behavior in male quail which implies that the male is the behaviorally "neutral" sex (*11*).

We find that the female zebra finch is the "neutral" sex with respect to sexual differentiation of the song system; early exposure of females to exogenous steroid induces masculinization of morphology and functional capacity for song. The cytoarchitecture of the telencephalic song nuclei RA and HVc is influenced by both DHT and E_2. The volume of RA was significantly larger ($P < .03$; Mann-Whitney U test) in both E_2- and DHT-females than in normal females. In E_2-females, RA reached an average volume of 0.137 mm^3 (range, 0.116 to 0.179 mm^3) and in DHT-females, 0.046 mm^3 (0.039 to 0.052 mm^3), which corresponds to an increase of 4.9-fold and 1.6-fold, respectively, over the volume of RA in normal gonadally intact females [0.028 mm^3 (0.021 to 0.036 mm^3)]. If E_2 and DHT affect independent ontogenetic processes (*12*), their combined effect should cause an eightfold increase in the size of RA, which corresponds to the volume of the male RA, 0.223 mm^3 (0.213 to 0.233 mm^3). The volume of HVc was influenced by both hormones in a similar fashion (Fig. 1).

In contrast to HVc and RA, DM is influenced solely by DHT. In DHT-females the volume of DM, 0.051 mm^3 (0.045 to 0.059 mm^3), was significantly larger ($P < .05$) than the volume of DM in control females, 0.028 mm^3 (0.032 to 0.047 mm^3). In E_2-females, the volume of DM, 0.039 mm^3 (0.032 to 0.046 mm^3), remained equivalent to that of the control.

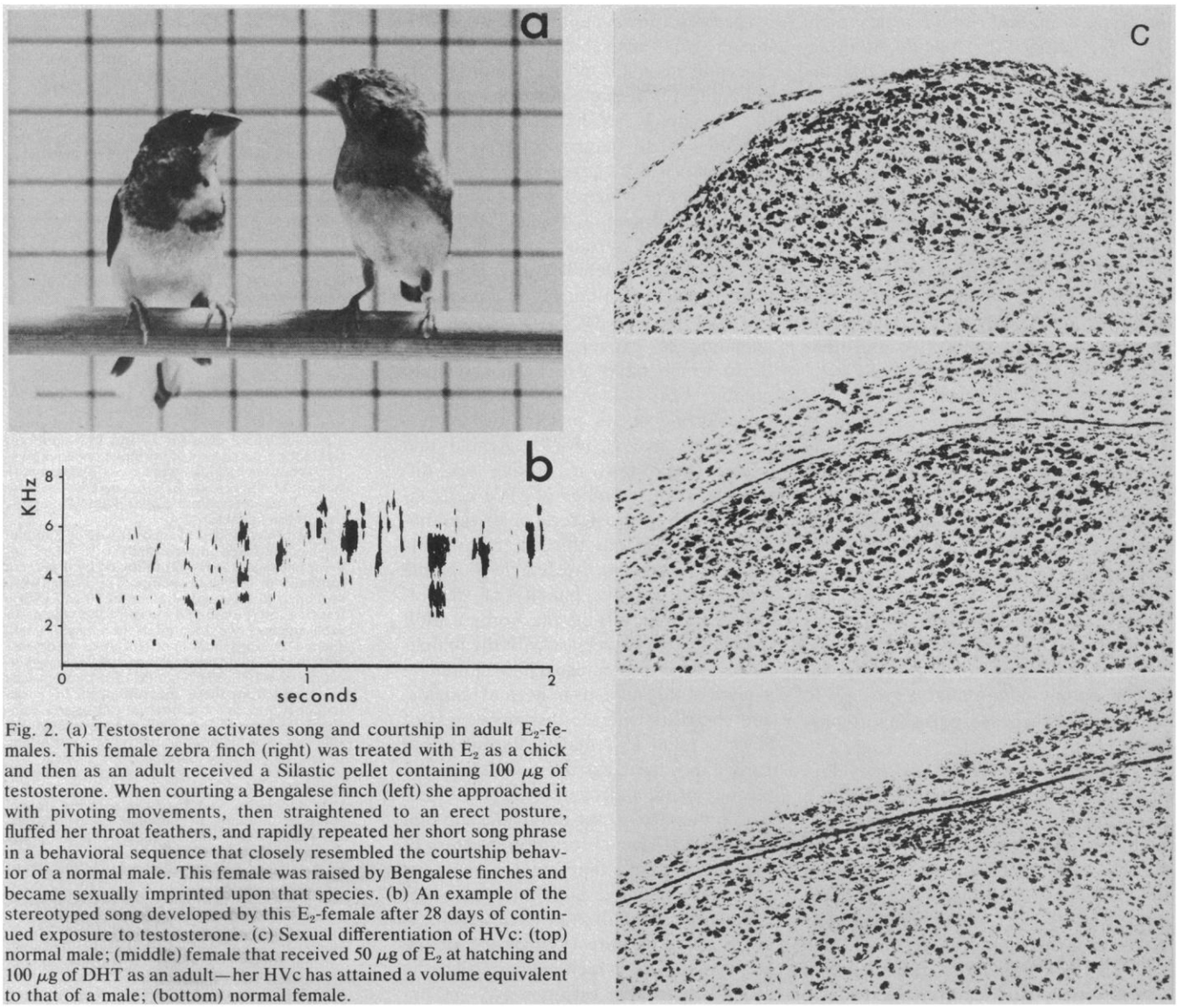

Fig. 2. (a) Testosterone activates song and courtship in adult E_2-females. This female zebra finch (right) was treated with E_2 as a chick and then as an adult received a Silastic pellet containing 100 μg of testosterone. When courting a Bengalese finch (left) she approached it with pivoting movements, then straightened to an erect posture, fluffed her throat feathers, and rapidly repeated her short song phrase in a behavioral sequence that closely resembled the courtship behavior of a normal male. This female was raised by Bengalese finches and became sexually imprinted upon that species. (b) An example of the stereotyped song developed by this E_2-female after 28 days of continued exposure to testosterone. (c) Sexual differentiation of HVc: (top) normal male; (middle) female that received 50 μg of E_2 at hatching and 100 μg of DHT as an adult—her HVc has attained a volume equivalent to that of a male; (bottom) normal female.

To examine whether or not the response of HVc, RA, or DM to exogenous steroid is confined to a limited period after hatching, we implanted Silastic pellets containing 100 μg of either E_2 or DHT into adult females with intact gonads and killed them 30 days later. After exposure to DHT, the brainstem nucleus DM of adult females attained a volume of 0.069 mm^3 (0.066 to 0.076 mm^3), which approximates the volume of DM in the male, 0.072 mm^3 (0.069 to 0.077 mm^3). In contrast, E_2 did not exert an effect on the volume of RA, HVc, or DM in adult females. Thus, the temporal constraints on the effect of E_2 appear more stringent than those on the effects of DHT.

The adult male's song is androgen-dependent (*13*), whereas exogenous androgen has never been seen to evoke song in normal adult female zebra finches (*14*). Adult E_2- or DHT-females did not sing without exogenous androgen despite masculinization of areas within their song system. Like the male, we thought that song in masculinized females might also be androgen-dependent. Adult females that had received E_2 or DHT implants as chicks were implanted with Silastic pellets containing 100 μg of either DHT or testosterone and housed individually in soundproof chambers. We then recorded their vocal behavior. Both testosterone and DHT activated song in E_2-females (Fig. 2), but androgen implanted DHT-females were never observed to sing. The E_2-females began to sing within 24 hours after initial exposure to androgen; these initial vocalizations were of unstable structure. Usually within 5 days several distinct acoustic elements developed and were sung with a regular temporal sequence. The highly stereotyped song of a testosterone implanted E_2-female (B303) emerged after 28 days of continuous androgen exposure (Fig. 2); this female also exhibited courtship behavior. The quality of B303's song (that is, its tempo, the acoustic definition of individual notes within it, and its stereotypy), was equivalent to the songs of male zebra finches in our colony. Continual exposure to androgen was necessary to support singing.

Androgen-dependent song development by E_2-females is coincident with a second phase of androgen-induced morphological differentiation in HVc and RA. A dramatic effect of hormone on HVc and RA of E_2-females was revealed when the birds were killed 30 days after initial exposure to androgen (Figs. 1 and 2c). DHT appears more effective than testosterone at inducing an increase in the volume of these nuclei in the adult E_2-female. The volume attained by HVc in E_2-females that received testosterone (0.248 mm^3), and E_2-females that received DHT (0.308 mm^3), was significantly larger than that of HVc in E_2-females (0.128 mm^3). When the data for the volume of HVc in E_2-females that received either testosterone or DHT as adults were lumped together they differed significantly from the volume of HVc in E_2-females ($P < .02$). Androgen also increased the volume of RA in adult E_2-females. Similar treatment of normal, intact adult female zebra finches with exogenous DHT had no effect on the volume of either RA or HVc. This suggests that as a consequence of E_2-mediated sexual differentiation, RA and HVc have become sensitive to androgen in the adult.

We do not know whether hormone directly affects cells of HVc, RA, or DM, or whether these effects are secondary to sexual differentiation of other brain areas (expressed transsynaptically). Toran-Allerand (*15*) has shown that isolated pieces of neonatal mouse hypothalamus maintained in tissue culture can respond to exogenous sex hormones with enhanced neurite outgrowth; this area of outgrowth was localized to the region of the explant in which a subpopulation of steroid-concentrating cells could be identified by steroid autoradiography (*16*). In adult E_2-females and in normal adult female zebra finches, the brain nuclei that we find to be capable of responding to exogenous androgen are those which have also been shown to contain cells which concentrate [^{3}H]-testosterone or its metabolites in normal adult males (*17–19*). Arnold and Saltiel (*18*) report a striking sex difference in the number of HVc cells labeled by [^{3}H]testosterone or its metabolites: less than one-third as many cells become labeled in the female as in the male. This relative paucity of steroid-concentrating cells in the normal adult female's HVc correlates with the inability of exogenous androgen to stimulate growth of this nucleus in normal females, and contrasts with the responsiveness of HVc in adult E_2-females. We infer that early exposure to estrogen modulates the efficacy of an androgen receptor system within the HVc of the adult (*20*) and that this effect is crucial for differentiation of the song system's functional capacity for androgen-mediated activation of song. Cellular accumulation of [^{3}H]estradiol is not observed in the HVc or RA of the adult male (*19*), which suggests that the sensitive period to estrogen may end because of the disappearance of the estrogen-receptor system. In contrast, the lack of a limited sensitive period in the DM may imply the presence of an androgen receptor system both at hatching and in the adult (*17*).

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6. We wished to distinguish between androgen-mediated and estrogen-mediated differentiative processes. As testosterone may be aromatized to 17β-estradiol by an enzyme found in brain extracts of both birds [G. V. Callard, Z. Petro, K. J. Ryan, *Endocrinology* **103**, 2283 (1978)] and mammals [F. Naftolin, K. J. Ryan, I. J. Davies, V. V. Reddy, F. Flores, Z. Petro, R. J. White, Y. Takaoka, L. Wolin, *Recent Prog. Horm. Res.* **31**, 295 (1975)], we used an androgen, 5α-dihydrotestosterone, which is not aromatized to estrogenic metabolites in vitro [K. J. Ryan, *Acta Endocrinol.* **35** (Suppl. 51), 697 (1960)].
7. The DM lies dorsomedial to the nucleus mesencephalicus lateralis, pars dorsalis, beneath the floor of the tectal ventricle within the nucleus intercollicularis. The DM receives afferents from RA, and in turn projects ipsilaterally to the nucleus nervi hypoglossus, nXII (M. Gurney, unpublished observations).
8. A 1:5 (by weight) mixture of steroid and RTV 738 Silastic (Dow Corning) was extruded through a 21-gauge hypodermic needle to form a rope that contained 50 μg of steroid per millimeter. This was cured overnight at 37°C, and then 1-mm lengths of the steroid-Silastic rope were used for implants. The rate of hormone release from the pellet in vivo decays exponentially with a half-life of 3.3 days for E_2 and 15.5 hours for DHT; the blood levels of hormone are unknown. The zebra finch chicks were cross-fostered with Bengalese finches (*Lonchura striata*) because birds of this species are more reliable as parents than zebra finches.
9. The birds were killed by an overdose of Equithesin, and perfused intracardially with 0.13*M* sodium phosphate (*p*H 7.2) followed by 4 percent Formalin in the same buffer. The brains were sectioned in the sagittal plane at 30 μm with a freezing microtome. To measure the volume of each nucleus we located it in cresyl violet–stained sections and then traced its perimeter with the aid of a camera lucida at a final magnification of ×100. The volume of a nucleus was calculated from these measurements of cross-sectional area and the interval (90 μm) of sampling. Because of the small sample sizes available, we used the nonparametric Mann-Whitney U test to compare differences in nuclear volumes among different groups of birds at a significance level of .05.
10. It is interesting that E_2-males sing but do not court or attempt to copulate with females, whereas in DHT-males song, courtship, and copulatory behavior are intact.
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13. Song is activated in castrated males by testoster-

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Levels of Batrachotoxin and Lack of Sensitivity to Its Action in Poison-Dart Frogs (*Phyllobates*)

Abstract. *Batrachotoxin is present in remarkably high amounts in the skin of* Phyllobates terribilis. *Levels of batrachotoxin tend to be reduced when* P. terribilis *is maintained in captivity, but even after being confined for up to 6 years, these frogs were still at least five times more toxic than other* Phyllobates *species used by natives for poisoning blowgun darts. Batrachotoxin was not detectable in F_1 progeny reared to maturity in captivity. Nerve and muscle preparations from wild-caught frogs and from the nontoxic F_1 frogs were both insensitive to batrachotoxin. The regulatory site controlling sodium-channel activation and permeability appears to have been minimally altered to prevent interaction with batrachotoxin, but is still sensitive to other sodium conductance activators (veratridine, grayanotoxin) to which the frogs are not exposed naturally.*

Batrachotoxin (molecular weight 538), a complex steroidal alkaloid, is one of the most toxic small molecules known (*1*). It owes its toxicity to a selective and virtually irreversible interaction with the voltage-dependent sodium channels in nerve and muscle, leading to a frequency- and concentration-dependent membrane depolarization. Batrachotoxin has been detected in nature in five species of Neotropical frogs, and its seemingly unique occurrence in these frogs is evidence for a monophyletic origin of the redefined genus *Phyllobates* (Dendrobatidae) of lower Central America and northwestern South America (*2*).

Little is known of the biosynthesis of batrachotoxin and its congeners except that they (and nearly 100 other less toxic and structurally simpler dendrobatid alkaloids) (*3*) seem to be produced in granular "poison" glands that are morphologically similar throughout the Dendrobatidae (*4*). Radioactivity was not detected in dendrobatid skin toxins after the administration of labeled cholesterol, mevalonate, and acetate, which seemed likely precursors (*5*). Levels of the batrachotoxin alkaloids differ greatly among the species of *Phyllobates*. In population samples of Panamanian *P. lugubris* and Costa Rican *P. vittatus*, amounts range from undetectable to about 0.8 μg per frog (*6*). Levels are much higher in *P. aurotaenia*, *P. bicolor*, and the recently described *P. terribilis*, which are confined to western Colombia and which are the only frogs known to be used by natives as the source of poison for their blowgun darts (*2*). *Phyllobates terribilis* is by far the most toxic species; the skin of an adult (body length < 50 mm) contains up to 1.9 mg.

The presence of such an extraordinarily toxic substance in the granular skin glands and its secretion onto the skin after attack by a predator would appear to make self-intoxication a possibility. Vesicles in the skin glands presumably serve to store batrachotoxin and prevent its translocation (*4*), but, after release, the toxin should be readily reabsorbed, particularly when the delicate skin is abraded or punctured in the grasp of a predator.

Earlier it was found that whereas nerve and muscle from *P. aurotaenia* (*7*) are virtually insensitive to the action of batrachotoxin, these tissues are highly batrachotoxin-sensitive in *Rana pipiens* (*7*) and another dendrobatid frog, *Dendrobates histrionicus* (*8*). In *P. aurotaenia*, it appeared that the effector site for the toxin associated with voltage-dependent sodium channels was absent or modified, or that the site was desensitized through constant exposure to the toxin. In the present study, we measured the toxicity levels of two adult *P. terribilis* (*9*) reared in captivity and investigated the sensitivity of preparations of their nerve and muscle tissue to the action of batrachotoxin and two other sodium conductance activators, veratridine and grayanotoxin. We also measured skin levels of batrachotoxin in wild-caught *P. terribilis* after they were maintained in captivity for various periods. Finally, we investigated the batrachotoxin and veratridine sensitivity of nerve and muscle from a frog killed 6 years after capture. Our results raise questions concerning the control of toxin production and the levels maintained in the skin of poison-dart frogs.

The average batrachotoxin-homobatrachotoxin content of ten *P. terribilis*, killed at time of capture, was 1140 ± 144 μg (range, 700 to 1900 μg) (*2*, *6*). Eight other individuals from the same locality averaged 540 ± 50 μg (range, 400 to 600 μg) when killed 3 weeks to 1 year after capture—a decrease to 47 percent of the original mean value. After 3 years in captivity, two individuals contained 320 and 480 μg (28 and 42 percent of the value for the freshly caught frogs). Finally, after 6 years in captivity, one *P. terribilis* contained 250 μg, or 22 percent of the original average value. Even with such a reduction, this old captive still contained five times the amount of toxin normally present in its nearest relatives, *P. bicolor* and *P. aurotaenia* (*2*).

Thus, even after 6 years, captive *P. terribilis* still contain relatively large amounts of skin toxins. It seems highly unlikely that batrachotoxin could be stored in the secretory glands for so long; indeed, in this time one might expect complete turnover of the cells that comprise the granular skin glands. We conclude that the frogs continue to synthesize batrachotoxin in captivity, albeit at a reduced rate or at least with less accumulation. Whether lower levels of essential dietary factors (*10*), unnatural crowding, or other laboratory-induced stress—or lack of stress (*11*)—might account for the decrease is unknown.

Two *P. terribilis* reared in captivity were relatively nontoxic, which was quite unexpected considering the apparent continued production of toxins in the parental colony. Batrachotoxin and homobatrachotoxin, if present, were at levels below the limits of positive detection by either color reaction or mouse assay (< 0.05 μg per 100 mg of skin). The extracts were still pharmacologically active (*12*); however, it is uncertain whether this activity was due to the presence of batrachotoxins (at about 0.02 μg per frog) or some other substance. Thus the frogs reared in captivity either did not synthesize the toxins or did not do so at a rate sufficient for measurable amounts to

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