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AN UPTAKE SYSTEM FOR DIETARY ALKALOIDS IN POISON FROGS (DENDROBATIDAE)

JOHN W. DALY,[1] SHERRIE I. SECUNDA,[1] H. MARTIN GARRAFFO,[1] THOMAS F. SPANDE,[1] ANTHONY WISNIESKI[2] and JACK F. COVER JR[3]

[1]Laboratory of Bioorganic Chemistry, National Institutes of Health, Bethesda, MD 20892, U.S.A.; [2]Herpetology Department, Baltimore Zoo, Baltimore, MD 21217, U.S.A.; and [3]National Aquarium in Baltimore, Baltimore, MD, U.S.A.

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J. W. DALY, S. I. SECUNDA, H. M. GARRAFFO, T. F. SPANDE, A. WISNIESKI and J. F. COVER JR. An uptake system for dietary alkaloids in poison frogs (Dendrobatidae). *Toxicon* **32**, 657–663, 1994.—The skin of poison frogs (Dendrobatidae) contains a wide variety of alkaloids that presumably serve a defensive role. These alkaloids persist for years in captivity, but are not present in captive-raised frogs. Alkaloids fed to poison frogs (*Dendrobates*, *Phyllobates*, *Epipedobates*) are readily accumulated into skin, where they remain for months. The process can be selective; an ant indolizidine is accumulated, while an ant pyrrolidine is not. Frogs (*Colostethus*) of the same family, which do not normally contain alkaloids, do not accumulate alkaloids. Such an alkaloid uptake system provides a means of maintaining skin alkaloids and suggests that some if not all such 'dendrobatid alkaloids' may have a dietary origin.

INTRODUCTION

A WIDE range of alkaloids has been isolated from the skin of poison frogs of the family Dendrobatidae (DALY *et al.*, 1993). Few of the alkaloids were given trivial names, instead because of their large number most have been designated with bold face numbers, corresponding to their nominal mass, with code letters added to distinguish alkaloids with the same nominal mass (DALY *et al.*, 1978, 1987). Several classes of these 'dendrobatid alkaloids' appear to be unique to amphibians, including the batrachotoxins, the histrionicotoxins, the 5,8-disubstituted indolizidines, the 1,4-disubstituted quinolizidines, the gephyrotoxins, the 2,5-disubstituted decahydroquinolines, the pumiliotoxin–allopumiliotoxin–homopumiliotoxin complex, and epibatidine, while others, namely simple piperidines and pyrrolidines, 3,5-disubstituted indolizidines and 3,5-disubstituted pyrrolizidines, also occur in ants (JONES and BLUM, 1983), and the tricyclic coccinellines occur both in frogs (DALY *et al.*, 1993) and in beetles (AYER *et al.*, 1976). Recently, homobatrachotoxin was discovered in the skin and feathers of a bird (DUMBACHER *et al.*, 1992) and, thus, it is not unique, as previously thought, to dendrobatid frogs of the genus *Phyllobates*. The absence of alkaloids in captive-raised poison frogs (DALY *et al.*, 1980, 1992) indicates that environmental factors are necessary for alkaloid accumulation. A variety of treatments of captive-raised frogs (*Dendrobates auratus*) did not result in

detectable levels of skin alkaloids. These included stress due to frequent changes in terraria, bath treatments, visual threats or swabbing, and increased fluorescent lighting (DALY *et al.*, 1992, and unpublished results). Even after coexistence for 1 year and even breeding with wild-caught alkaloid-containing frogs (*Dendrobates auratus*), captive-raised frogs did not contain detectable levels of skin alkaloids, a result arguing against an essential symbiotic microorganism. Thus, dietary factors and/or dietary alkaloids remained the most likely environmental factors important to alkaloid accumulation in the skin of dendrobatid frogs. Either essential precursors or cofactors, or the alkaloids themselves, might be obtained from the rain forest arthropods on which dendrobatid frogs feed. Therefore, the ability of dendrobatid frogs to accumulate alkaloids from the diet into the skin was examined.

METHODS

The protocol for separation and analysis of alkaloids from skin extracts of frogs has been refined over the years and now allows identification and quantitative characterization of constituents in an alkaloid fraction from a single frog skin or less by gas chromatographic analysis. The methodology used in the present study is essentially as described (DALY *et al.*, 1992, 1993). In brief, the wet weight of skin was determined, followed by cutting into small pieces and maceration in a mortar and pestle three times, each time with a 20-fold excess of methanol. The combined methanol extracts were diluted with an equal volume of water. The aqueous methanol was extracted three times, each time with 1 vol. of chloroform. The combined chloroform layers were concentrated to a small volume of about 1 ml and restored to the original volume with *n*-hexane. The hexane was added to reduce the solubility of alkaloid hydrochlorides in the organic phase during partition with aqueous HCl. The *n*-hexane, containing a small amount of chloroform, was extracted three times, each time with 0.5 vol. of 0.1 N HCl. The combined 0.1 N HCl fractions were adjusted to pH 9.0 with 1 N aqueous ammonia, followed by re-extraction three times, each time with 0.5 vol. of chloroform. The combined chloroform extracts were dried with anhydrous Na_2SO_4 and evaporated to dryness at 30°C *in vacuo* with a water-aspirator. Evaporation was done carefully since many of the alkaloids have appreciable volatility. The resulting alkaloid residue was dissolved in sufficient methanol so that 100 μl of this alkaloid fraction corresponded to 100 mg of the original wet weight of skin. The relative amounts of alkaloids were assessed quantitatively by flame-ionization gas chromatography on a 6-foot 1.5% OV-1 packed column programmed at 10°C/min from 150°C to 280°C with a flow rate of 30 cm^3/min helium. Representative gas chromatograms for alkaloid fractions from skins of wild-caught dendrobatid frogs have been shown in previous publications (DALY *et al.*, 1987, 1990, 1991, 1993 and references therein; see also Fig. 1A). Identification of alkaloids was by gas chromatography–mass spectrometry and gas chromatography–infrared spectroscopy as described in prior publications (see Daly *et al.*, 1987, 1992, 1993).

Wingless fruit flies were raised on standard *Drosophila* medium (Carolina Biological Supply Co., Burlington, NC, U.S.A.). Before feeding to frogs, the fruit flies were dusted with a vitamin–mineral powder (Nekton-Rep from Nekton, Clearwater, FL, U.S.A.), which contained 1% or less by weight of one or more alkaloids. The alkaloids used were isolated from skin extracts of dendrobatid frogs (see DALY *et al.*, 1987), except for synthetic indolizidine **209B** which was provided by Dr A. B. HOLMES (Cambridge University, U.K.). Feeding of frogs with such dusted fruit flies was continued for up to 6 months. Frogs were then sacrificed and alkaloid fractions from skin extracts were prepared and analyzed by gas chromatography as described above. In some cases, frogs were maintained on a normal diet for periods of up to 5 months after alkaloid feeding was stopped. The frogs used in these experiments were either wild-caught or were raised to adulthood in captivity at the National Aquarium in Baltimore. Most experiments were with captive-raised *Dendrobates auratus*, but a limited number of experiments were with wild-caught *Dendrobates auratus*, captive-raised *Phyllobates bicolor* and *Epipedobates tricolor*, and wild-caught *Colostethus* species.

Ants (*Monomorium pharaonis*) were raised in colonies, fed on honey and crickets (Flucker's Cricket Farm Inc., Baton Rouge, LA, U.S.A.). These ants were fed to frogs. The frog's diet of ants was supplemented occasionally with wingless fruit flies.

RESULTS AND DISCUSSION

Virtually all dendrobatid frogs of the genera *Phyllobates*, *Dendrobates*, *Epipedobates* and *Minyobates* have significant levels of skin alkaloids as detected by gas chromatographic analysis (DALY *et al.*, 1987). The parent stock of the captive-raised *D. auratus* used in the present feeding experiments was no exception. Gas chromatographic traces of alkaloid fractions from four different individuals are shown in Fig. 1. All four contain a range of

pyrrolizidines, indolizidines, quinolizidines, decahydroquinolines, and histrionicotoxins. Levels of alkaloids in skin of dendrobatid frogs have been found to vary significantly for different individuals. This variation is illustrated by one individual with very high levels of about 200 μg total alkaloids per 100 mg skin extract (Fig. 1A), while another individual had very low levels (Fig. 1C). Most individuals have levels intermediate to these extremes

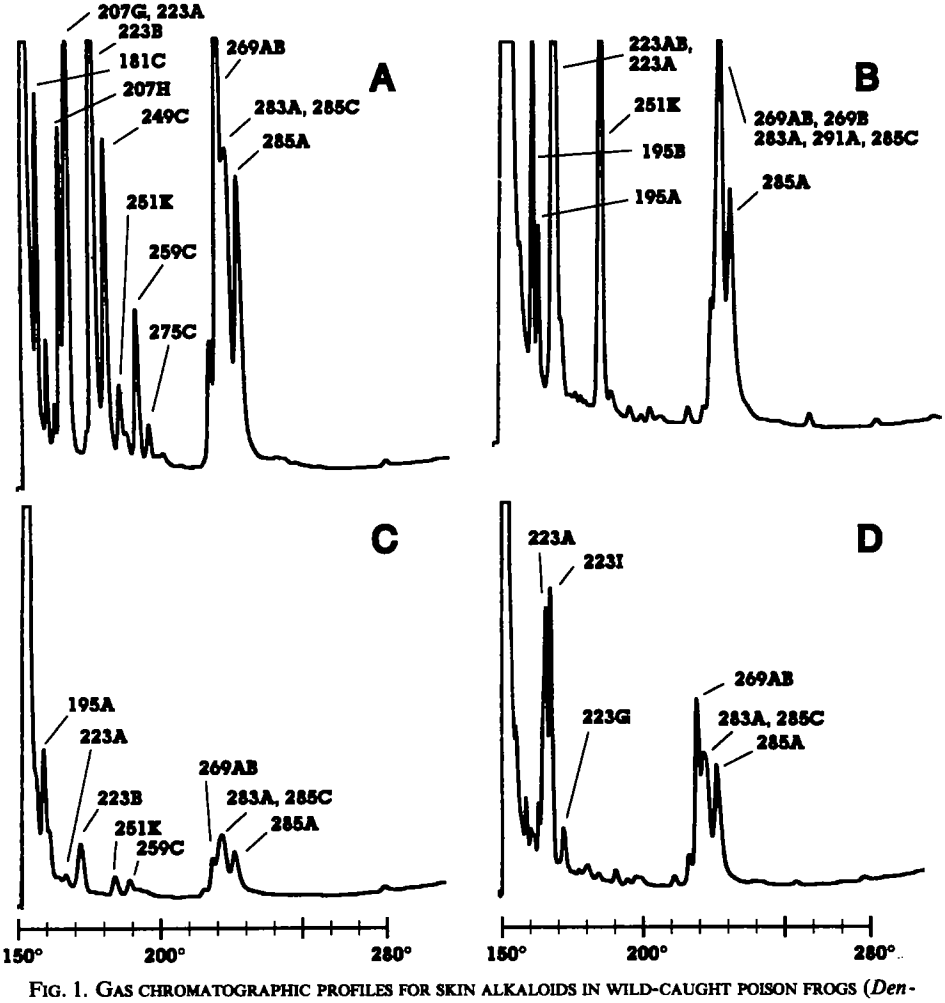

FIG. 1. GAS CHROMATOGRAPHIC PROFILES FOR SKIN ALKALOIDS IN WILD-CAUGHT POISON FROGS (*Dendrobates auratus*).

Skin extracts from individual specimens from Bribri, Limon Province, Costa Rica, were made in the field in June 1990 (A,B,C) or after 2 years in captivity (D). A chromatogram for another specimen from this site (June 1989) is depicted as Fig. 2B in DALY *et al.* (1992). The alkaloids are comprised mainly of pyrrolizidines (**223B**, **251K**), indolizidines (**195B**, **223AB**, **223A**, **223I**), quinolizidines (**249C**, **259C**, **275C**), decahydroquinolines (**195A**, **269AB**, **269B**) and histrionicotoxins (**283A**, **285A**, **285C**, **291A**). Homopumiliotoxins (**207H**, **223G**) and allopumiliotoxins (**267A**) also occur. (For structures see DALY *et al.*, 1993.) The gas chromatograms were obtained with a 6-foot (2 mm i.d.) 1.5% OV-1-packed column with a flame ionization detector and a flow rate of 30 cm^3/min helium. A sample of 2 μl of a methanolic alkaloid fraction equivalent to 2 mg wet weight skin was injected at a column temperature of 150°C. After the solvent maximum had passed (0.3 min), the column was programmed to 280°C at 10°C/min. For further details and gas chromatographic mass spectral identification of alkaloids, see DALY *et al.* (1987, 1992, 1993).

(Fig. 1B, see also chromatogram in DALY *et al.*, 1992). The fourth wild-caught individual was maintained in captivity for 2 years and still has significant levels (Fig. 1D), as has been the case for other wild-caught dendrobatid frogs maintained in captivity for 1–4 years (see below). In all cases, it is unknown what levels of alkaloids were present when that particular individual frog was originally captured. Frogs raised in captivity have no detectable levels of alkaloids even by sensitive gas chromatographic mass spectral analysis (DALY *et al.*, 1992).

Captive-raised frogs (*D. auratus*, parent stock from Bribri, Limon Province, Costa Rica, see Fig. 1) that were fed fruit flies dusted with alkaloid-containing powder were found to accumulate alkaloids readily into skin. Histrionicotoxins, 2,5-disubstituted decahydroquinolines, 3,5-disubstituted pyrrolizidines, 3,5- and 5,8-disubstituted indolizidines, and 1,4-disubstituted quinolizidines were all accumulated into skin (Fig. 2A, B, C and data not shown). After feeding fruit flies dusted with allodihydrohistrionicotoxin (**285C**) for 6 months a very high accumulation was found (Fig. 2A). Such accumulation resulted in levels similar to the highest levels of any alkaloid in wild-caught frogs (compare to Fig. 1A). Much lower accumulations resulted in the shorter-term experiments. Indeed, levels in most experiments were similar to those depicted for a frog fed a mixture of alkaloids for 3 months, followed by 2 months on a diet of undusted fruit flies (Fig. 2B). A wide range of alkaloids fed as a mixture for only 2 weeks resulted in significant, but low accumulation (Fig. 2C). A pyrrolidine **197B** (*cis*-2-butyl-5-pentylpyrrolidine) present in the mixture of alkaloids, however, was not detected. Similarly, in another feeding experiment a piperidine (*cis*-2-methyl-6-undecylpiperidine) was not accumulated (Fig. 2B). Pumiliotoxin (**307A**) and allopumiliotoxins (e.g. **267A**) appeared to accumulate only in trace amounts. The diet-derived alkaloids persisted in skin for several months (Fig. 2B and data not shown). Frogs fed for 2 months with decahydroquinoline **195A** had significant levels in skin, while none could be detected in liver or muscle (data not shown). Wild-caught *D. auratus* also had no alkaloids in muscle or internal organs. In experiments in which wild-caught *D. auratus* (Ancon Hill, Panama City, Panama) were fed a synthetic 5,8-disubstituted indolizidine **209B**, the ratio of the dietary **209B** alkaloid in skin to that of other naturally occurring alkaloids, specifically decahydroquinoline **195A**, was essentially the same (8:3 to 8:4) in a frog sacrificed at the end of the 1-month feeding with **209B** and in frogs sacrificed 3, 4 and 5 months after cessation of feeding dietary **209B** (data not shown). Thus, diet-derived alkaloids appear to be maintained as well as the 'natural' alkaloids originally present in wild-caught frogs.

Ants of the myrmicine species *Monomorium pharaonis* produce two major alkaloids, namely a 3,5-disubstituted indolizidine, momomorine-I (5Z,9Z-3-butyl-5-methylindolizidine), and trans-2-heptyl-5-(5-hexenyl)pyrrolidine (RITTER *et al.*, 1975). A 3-butyl-5-methylindolizidine has been detected in skin of wild-caught dendrobatid frogs and designated indolizidine **195B**, but the stereochemistry of **195B** from frogs usually differs in stereochemistry from that of monomorine-I (DALY *et al.*, 1993). After feeding frogs the *M. pharaonis* for 7 weeks, there was a significant accumulation of the indolizidine monomorine-I into skin, while not even a trace of the pyrrolidine was detected (Fig. 2D). In the ants the pyrrolidine is present at about five-fold higher levels than the indolizidine (data not shown). Two minor alkaloids, apparently 3-hexenyl-5-methylindolizidines, also accumulated in the frog skin (Fig. 2D).

The present feeding experiments demonstrate the remarkable ability of a dendrobatid frog, namely *D. auratus*, to accumulate a range of dietary 'dendrobatid alkaloids' into the skin. Natural alkaloids in wild parent stock were mainly 3,5-disubstituted pyrrolizidines,

5,8-disubstituted indolizidines, 1,4-disubstituted quinolizidines, 2,5-disubstituted decahydroquinolines, and histrionicotoxins (Fig. 1A–D). Pumiliotoxins were very minor constituents. Levels and profiles can vary considerably in different individuals.

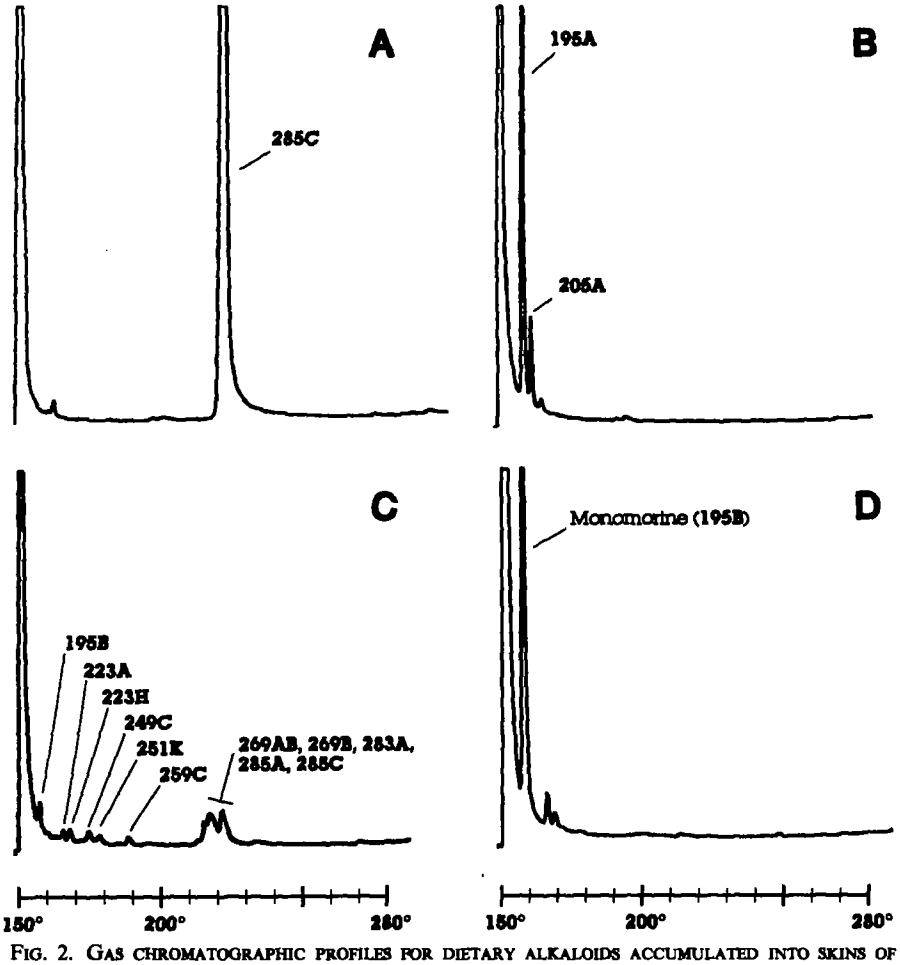

FIG. 2. GAS CHROMATOGRAPHIC PROFILES FOR DIETARY ALKALOIDS ACCUMULATED INTO SKINS OF CAPTIVE-RAISED POISON FROGS (*Dendrobates auratus*).

A. Alkaloid fraction from a captive-raised *Dendrobates auratus* fed fruit flies dusted with powder containing 7 mg allodihydrohistrionicotoxin (**285C**) per g vitamin-mineral powder for 6 months. A similar accumulation was found in a second frog. B. Alkaloid fraction from a captive-raised *Dendrobates auratus* fed fruit flies dusted with powder containing 6 mg decahydroquinoline **195A**, 2 mg 5,8-disubstituted indolizidine **205A** and 5 mg *cis*-2-methyl-6-undecylpiperidine per g powder for 3 months, followed by 2 months on undusted fruit flies. The piperidine was not accumulated into skin. C. Alkaloid fraction from a captive-raised *Dendrobates auratus* fed fruit flies dusted with powder containing a complex mixture of alkaloids isolated from 20 *Dendrobates pumilio* (Bribri, Limon Province, Costa Rica) with an estimated 4 mg of alkaloid per g powder for 2 weeks. The alkaloid profile is virtually identical to that which was fed, namely a mixture of pyrrolizidines (**223H**, **251K**), indolizidines (**195B**, **223A**), quinolizidines (**249C**, **259C**), decahydroquinolines (**269AB**, **269B**), and histrionicotoxins (**283A**, **285A**, **285C**). D. Alkaloid fraction from a captive-raised *Dendrobates auratus* fed ants (*Monomorium pharaonis*), which contain the indolizidine monomorine-I (5Z,9Z-3-butyl-5-methylindolizidine) and *trans*-2-heptyl-5-(5-hexenyl)pyrrolidine (1:5 ratio), for 7 weeks. The pyrrolidine was not accumulated into skin. The gas chromatograms were obtained as described in the legend to Fig. 1.

Quantitative analysis of the present results was not possible, since for each frog the total intake of alkaloids, provided either from dusted fruit flies or from ants, could not be ascertained. In some cases, remarkable accumulations occurred (Fig. 2A), while in others accumulations were lower (Fig. 2B–D, and data not shown). The ratio of alkaloids in the dusting powder was usually quite similar to the ratio of alkaloids subsequently found in the frog skin after feeding mixtures of decahydroquinolines, pyrrolidines, indolizidines, quinolizidines and histrionicotoxins, suggesting a rather non-specific uptake for lipophilic alkaloids. It is noteworthy that the 2,5-disubstituted pyrrolidines and a 2,6-disubstituted piperidine were not accumulated, since in certain dendrobatid species such alkaloids, particularly pyrrolidine **197B**, can be significant components of the skin alkaloids (DALY *et al.*, 1987). The existence of an efficient uptake system in dendrobatid frogs might, since frogs eat their own skin when shedding (WELDON *et al.*, 1993), explain the long-term retention of high levels of alkaloids in wild-caught dendrobatid frogs maintained in captivity for years. Retention of alkaloids in captivity has been demonstrated for *D. auratus* (2–3 years, skin alkaloids: mainly decahydroquinolines, histrionicotoxins; see Fig. 1D), *D. azureus* (3 years, skin alkaloids: mainly indolizidines, decahydroquinolines, histrionicotoxins and allopumiliotoxin **267A**), *D. lehmanni* (2 years, skin alkaloids: mainly quinolizidine **275A**, pumiliotoxins and allopumiliotoxins), *D. tinctorius* (2–4 years, skin alkaloids: mainly indolizidines, quinolizidine **231B**, pyrrolizidine oxime **236**, decahydroquinolines and histrionicotoxins), *Epipedobates trivittatus* (1.5 years, skin alkaloids: mainly pyrrolizidine oxime **236**, decahydroquinolines and histrionicotoxins), and *Phyllobates terribilis* (3–6 years, skin alkaloids: mainly batrachotoxins, see DALY *et al.*, 1980).

Virtually all of the above feeding experiments were with the readily available captive-raised *D. auratus*. In more limited experiments, alkaloids (decahydroquinoline **195A**, 5,8-disubstituted indolizidines **205A** and **209B** and histrionicotoxin **285C**) were shown to be accumulated into skin of captive-raised *Phyllobates bicolor* and *Epipedobates tricolor* (data not shown). Captive-raised *P. bicolor* also were shown to accumulate very low levels of batrachotoxinin A in skin after eating fruit flies dusted with powder containing 0.5% batrachotoxinin A for 7 weeks. The batrachotoxinin A, a potential precursor for batrachotoxin/homobatrachotoxin in such frogs, was detectable at low levels in skin for at least 7 weeks, but there was no conversion to batrachotoxin/homobatrachotoxin (data not shown). After 6 months no alkaloids were detectable.

All of the dendrobatid frogs shown to accumulate dietary alkaloids into skin are frogs of genera that have skin alkaloids in the wild, namely *Dendrobates*, *Epipedobates* and *Phyllobates*. Specimens of the fourth alkaloid-containing dendrobatid genus *Minyobates* were not available. Frogs of the dendrobatid genus *Colestethus* do not contain skin alkaloids. Feeding experiments with Panamanian *Colostethus talamancae* and *C. inguinalis* with a mixture of 5,8-disubstituted indolizidine **209B**, decahydroquinoline **195A** and histrionicotoxin **285C** for 5 weeks did not result in detectable accumulation of skin alkaloids (data not shown). An identical feeding schedule with this mixture did result in accumulation of these alkaloids into skin of captive-raised *D. auratus*, and *P. bicolor* (data not shown, see Fig. 2B).

Although limited in scope, the present study demonstrates a remarkable system for accumulating dietary alkaloids into the skin of dendrobatid frogs. The uptake is present in frogs (*Dendrobates*, *Phyllobates* and *Epipedobates*) that are characterized in the wild as alkaloid-containing, and is absent in non-alkaloid-containing dendrobatids (*Colostethus*). The system accumulates a number of alkaloids known from dendrobatid frogs, namely

pyrrolizidines, indolizidines, quinolizidines, decahydroquinolines, histrionicotoxins, and batrachotoxins. The system does not appear to accumulate pyrrolidines or piperidines and appears to accumulate pumiliotoxins poorly. Such a system provides the poison frogs of this family with a means to accumulate alkaloids into skin from dietary sources, and to recycle alkaloids after eating shed skin. The results do not fully answer the question of the source of 'dendrobatid alkaloids' and the absence of such alkaloids in captive-raised frogs. But the results do suggest that many of the dendrobatid alkaloids might derive from dietary sources. Certainly, 3,5-disubstituted pyrrolizidines and 3,5-disubstituted indolizidines occur in ants (JONES and BLUM, 1983) and coccinellines occur in beetles (AYER *et al.*, 1976). The challenge remains to demonstrate what, if any, insects might contain the histrionicotoxins, pumiliotoxins, decahydroquinolines, 5,8-disubstituted indolizidines, 1,4-disubstituted quinolizidines and other unique 'dendrobatid' alkaloids, since these are as yet unknown in nature except for in amphibian skins.

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REFERENCES

AYER, W. A., BENNETT, M. J., BROWNE, L. M. and PURDHAM, J. T. (1976) Defensive substances in *Coccinella transversoguttata* and *Hippodamia caseyi*, lady bugs indigenous to western Canada. *Can. J. Chem.* **54**, 1807–1813.

DALY, J. W., BROWN, G. B., MENSAH-DWUMAH, M. and MYERS, C. W. (1978) Classification of skin alkaloids from neotropical poison-dart frogs (Dendrobatidae). *Toxicon* **16**, 163–188.

DALY, J. W., MYERS, C. W., WARNICK, J. E. and ALBUQUERQUE, E. X. (1980) Levels of batrachotoxin and lack of sensitvity to its action in poison-dart frogs (*Phyllobates*). *Science* **208**, 1383–1385.

DALY, J. W., MYERS, C. W. and WHITTAKER, N. (1987) Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the amphibians. *Toxicon* **25**, 1023–1095.

DALY, J. W., SECUNDA, S. I., GARRAFFO, H. M., SPANDE, T. F., WISNIESKI, A., NISHIHIRA, C. and COVER, J. F., JR (1992) Variability in alkaloid profiles in neotropical poison frogs (Dendrobatidae): genetic versus environmental determinants. *Toxicon* **30**, 887–898.

DALY, J. W., GARRAFFO, H. M. and SPANDE, T. F. (1993) Amphibian alkaloids. In: *The Alkaloids*, Vol. 43, Chapter 3, pp. 185–288 (CORDELL, G. A., Ed.). San Diego: Academic Press.

DUMBACHER, J. P., BEEHLER, B. M., SPANDE, T. F., GARRAFFO, H. M. and DALY, J. W. (1992) Homobatrachotoxin in the genus *Pitohui*: chemical defense in birds? *Science* **258**, 799–801.

JONES, T. H. and BLUM, M. S. (1983) Arthropod alkaloids: distribution, functions, and chemistry. In: *Alkaloids, Chemical, and Biological Perspectives*, Vol. 1, Chapter 2, pp. 33–84 (PELLETIER, S. W., Ed.). New York: Wiley.

RITTER, F. J. and PERSOONS, C. J. (1975) Recent developments in insect pheromone research, in particular in the Netherlands. *Netherland J. Zool.* **25**, 261–275.

WELDON, P. J., DEMETER, B. J. and ROSSCOE, R. (1993) A survey of shed skin-eating (dermatophagy) in amphibians and reptiles. *J. Herpet.* **27**, 219–228.