Biochemical Pharmacology, Vol. 20, pp. 2555–2559. Pergamon Press, 1971. Printed in Great Britain

BIOSYNTHESIS OF CHOLESTEROL AND CHOLESTEROL ACETATE IN DENDROBATID ARROW POISON FROGS

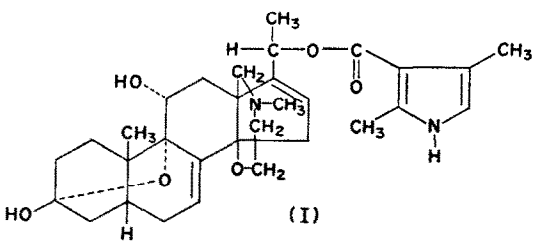
DAVID F. JOHNSON and JOHN W. DALY

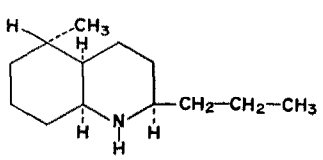
National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md., U.S.A.

(*Received* 10 *October* 1970; *accepted* 29 *January* 1971)

Abstract—The skin of neotropical poison frogs of the genus *Dendrobates* and *Phyllobates* presents a series of unique poisons containing decahydroquinoline and perhydrocyclopentanophenanthrene nuclei respectively. Examples of each class are pumiliotoxin C (2-*n*-propyl-5-methyl-*cis*-decahydroquinoline) and batrachotoxin. The biosynthesis of these poisons has now been investigated in *Dendrobates pumilio*, *D. auratus* and *Phyllobates aurotaenia*, using as possible precursors radioactive cholesterol, mevalonate and acetate. Even over periods of up to 14 weeks, significant incorporation of radioactivity into the poisons was not observed. Both acetate and mevalonate were, however, converted to cholesterol and cholesterol acetate.

THE NEOTROPICAL family of dendrobatid frogs has been found to contain unusual alkaloids as yet unknown from other natural sources. The extremely potent poison of the Colombian arrow poison frog, *Phyllobates aurotaenia*, has been identified as batrachotoxin (I, Fig. 1), a steroidal alkaloid with unique effects on ion permeability.[2] Frogs of the related genus, *Dendrobates*, contain poisonous decahydroquinoline alkaloids of which the simplest member is pumiliotoxin C (II, Fig. 1).[3] Nothing is known of the biosynthetic pathways involved in the formation of these novel poisons.*





(II)

FIG. 1. Structures of poisonous alkaloids from dentrobatid arrow-poison frogs. (I) Batrachotoxin; (II) pumiliotoxin C.

*Note added in proof: A third class of novel alkaloids, the histrionicotoxins have now been isolated from another species of dendrobatid frog (J. W. DALY, I. KARLE, C. W. Myers, T. TOKUYAMA, J. A. WATERS and B. WITKOP, *Proc. natn. Acad. Sci. U.S.A.*, in press).

Earlier studies had shown that cholesterol can serve as a precursor of poisons in certain amphibians as for example with the cardiotoxic bufodienolides[4–7] of toads (*Bufo*) and the neurotoxic samandarine alkaloids[8] of the European fire salamander (*Salamandara maculosa*). The reports on biosynthesis of bufodienolides from cholesterol in toads are, however, conflicting. Porto and Gros[4,5] found that mevalonate and pregnenolone were inefficient precursors of the bufodienolides. Chiadao and Osuch[6] reported a small but detectable amount of marinobufagin-^{14}C after injection of radioactive cholanates, but found that cholesterol-^{14}C was a very poor precursor. This is in contrast to a 2 per cent conversion of cholesterol to bufodienolides reported by Siperstein *et al.*[7] For the present studies on the biosynthesis of the poison alkaloids in dendrobatid frogs, radioactive cholesterol, mevalonate and acetate were administered by intraperitoneal injection to *Dendrobates pumilio*, *D. auratus* and *Phyllobates aurotaenia* for an extended period of time, and the radioactivity in skin extracts was analyzed for incorporation into the alkaloid poisons.

MATERIALS AND METHODS

Experiments in vivo. Frogs collected in Panama (*D. auratus*, *D. pumilio*) and Colombia (*P. aurotaenia*) were maintained in terrariums in laboratories at the National Institutes of Health and were fed fruit flies and *Tenebrio* larvae *ad lib*. They were injected intraperitoneally at 1-week intervals for a total of 6 weeks with 50 μl of solvent (15% ethanol in water for the cholesterols and water for mevalonate and acetate) containing cholesterol-4-^{14}C, cholesterol-26-^{14}C, *dl*-mevalonic acid-2-^{14}C or sodium acetate-2-^{14}C. The radioactive compounds were from commercial sources. Injections with sodium acetate-2-^{14}C were carried out for 14 weeks. At the end of the period of injection, frogs were sacrificed and skinned. The skins were minced and suspended in methanol overnight. The methanol extracts were decanted, filtered through sintered glass filters, concentrated to a small volume, and mixed with 3 × 50 ml $CHCl_3$ and 50 ml water. The aqueous layer was re-extracted with 3 × 50 ml $CHCl_3$. The combined $CHCl_3$ extracts were then extracted with 3 × 50 ml of 0·5 N HCl. The remaining $CHCl_3$ extract containing the neutral and acidic fraction was evaporated to dryness under vacuum. The acidic aqueous phase was made basic with 1 N NH_4OH and extracted with $CHCl_3$ to give a basic fraction. Each fraction, including the original and final aqueous phases, was counted in a scintillation spectrometer.

Thin-layer chromatography (*TLC*). Aliquots of the various residues were analyzed on silica gel-G thin-layer chromatoplates using three solvent systems: $CHCl_3$–MeOH (9:1); benzene–ethyl acetate (3:1); and cyclohexane–ethyl acetate (3:2). Radioactive zones were detected in a strip scanner and mobilities were compared with standards.

RESULTS

Table 1 shows the number of frogs injected, species, the total radioactivity of each substrate used in the studies *in vivo*, and the distribution of total radioactivity in the neutral and basic fractions for each series of experiments. The per cent of total injected radioactivity recovered in the neutral fraction after injection of cholesterol-4-^{14}C in *P. aurotaenia* was considerably higher than in the similar experiment with *D. auratus*.

The neutral fraction yielded sufficient radioactive material for detailed analysis. Each of the neutral extracts from the *in vivo* injections of mevalonate-2-^{14}C, sodium

TABLE 1. INCORPORATION *in vivo* OF LABELED PRECURSORS INTO NEUTRAL AND BASIC FRACTIONS PREPARED FROM SKIN BY METHANOL EXTRACTION AND SOLVENT PARTITIONS BETWEEN CHLOROFORM–AQUEOUS ACID–BASE

Substrate	(counts/min/μmole $\times 10^6$)	No. of frogs	Species	Total radioactivity injected (counts/min)	Neutral fraction		Basic fraction	
					Total radioactivity (counts/min)	% Total radioactivity injected	Total radioactivity (counts/min)	% Total radioactivity injected
Cholesterol-4-^{14}C	34·9	3	*D. auratus*	$3{\cdot}6 \times 10^6$	110,800	3	0	
Cholesterol-26-^{14}C	26·9	3	*D. auratus*	36×10^6	491,000	14	0	
Cholesterol-4-^{14}C	89·1	3	*P. aurotaenia*	$1{\cdot}4 \times 10^6$	557,200	39	800	0·1
Mevalonate-2-^{14}C	2·1	7	*D. pumilio*	$2{\cdot}1 \times 10^7$	852,500	4	4100	0·02
Mevalonate-2-^{14}C	5·4	3	*P. aurotaenia*	$3{\cdot}5 \times 10^6$	335,600	10	5000	0·1
Acetate-2-^{14}C	1·2	12	*D. pumilio*	1×10^8	155,000	0·2	17,200	0·02

TABLE 2. FORMATION OF CHOLESTEROL AND CHOLESTEROL ACETATE FROM LABELED PRECURSORS IN DENDROBATID (THIN-LAYER CHROMATOGRAHPIC ANALYSIS OF THE NEUTRAL FRACTION FROM SKIN)

Substrate	Species	Cholesterol-^{14}C		Cholesterol acetate-^{14}C		Ratio choles./choles. ac.
		% Total radioactivity injected	Total radioactivity (counts/min)	% Total radioactivity injected	Total radioactivity (counts/min)	
Cholesterol-4-^{14}C	*D. auratus*	2·1	73,000	0·9	34,000	2·2
Cholesterol-26-^{14}C	*D. auratus*	11	397,000	3	87,000	4·6
Cholesterol-4-^{14}C	*P. aurotaenia*	18	266,000	21	290,000	0·9
Mevalonate-2-^{14}C	*D. pumilio*	3·3	705,000	0·7	138,000	5·1
Mevalonate-2-^{14}C	*P. aurotaenia*	5	165,000	5	165,000	1·0
Acetate-2-^{14}C	*D. pumilio*	0·15	112,000	0·05	37,000	3·0

acetate-2-^{14}C, cholesterol-4-^{14}C and cholesterol-26-^{14}C was analyzed by TLC in the solvent systems described under Methods. Three zones of radioactivity were found on TLC in $CHCl_3$–MeOH (9:1) for each substrate. The two least polar compounds in this system had the same mobility as standard cholesterol and cholesterol acetate. Their identity was established by comparison with standards in two other solvent systems and by conversion of each into either free or acetylated compounds. The more polar third constituent was present in only a minor amount (2%) and was not identified. Table 2 shows the relative amount of free versus acetylated cholesterol in each experiment. From the standpoint of comparative biochemistry, it is of interest that the per cent of acetylated cholesterol is significantly higher in the experiment with *P. aurotaenia* than with *D. auratus* or *D. pumilio*.

Negligible amounts of radioactivity were found in the aqueous phases of the extraction procedure. Thus, only 0·1–0·2 per cent of the radioactivity in the mevalonate experiments was found in the original aqueous discard and similar small amounts were found in the cholesterol-^{14}C experiments. Despite the large total amount of radioactive sodium acetate used, only 0·2 and 0·01 per cent, respectively, were found in the aqueous phases.

The basic fraction, which contains the alkaloid poisons, presented very low amounts of radioactivity (Table 1). Thin-layer analysis of these fractions indicated that they contained small amounts of radioactive cholesterol and cholesterol acetate carried over from the neutral fraction during extraction. No radioactivity could be detected in the alkaloid poisons by thin-layer chromatographic analysis. Recovery of batrachotoxin, pumiliotoxin and related compounds through the extraction procedure and thin-layer chromatography is greater than 75 per cent.[1,3]

DISCUSSION

The toxic alkaloids isolated from skin extracts of various dendrobatid frogs are unique to this group of frogs and have, in all likelihood, been developed for use as a chemical defense against predators. Because of unique structural features and possible phyllogenetic implications, their biosynthesis is of some interest. Nothing was known of the turnover rate of these poisons or of the biosynthetic pathway involved in their formation. In the case of *P. aurotaenia*, batrachotoxin had been detected both in young frogs and in adults, but not in tadpoles (J. Daly, unpublished results). The present investigation had employed radioactive cholesterol, mevalonate and acetate as potential precursors. During the course of these experiments, incorporation of radioactivity from these precursors into the alkaloids was not detected. These negative results indicate either that: (1) none of the compounds tested is a precursor; (2) the precursors did not reach the skin glands that are presumably the site of synthesis of the alkaloids or (3) the rate of formation and turnover of the alkaloids is extremely slow. It seems quite unlikely that mevalonate or cholesterol, or both, would not serve as precursors for the steroidal alkaloids of the batrachotoxin class and that acetate would not serve as a precursor for the pumiliotoxin alkaloids. In addition, the high incorporation of radioactive cholesterol, mevalonate and acetate into skin cholesterol and cholesterol acetate demonstrates that the precursors are indeed rather selectively concentrated in the skin. It is therefore most probable that biosynthesis of these toxins has not been demonstrated because their rate of formation and turnover in the adult frog is extremely

slow. Preliminary studies on biosynthesis of these alkaloids *in vitro* with skin minces have also been unsuccessful.

Cholesterol is found as a major constituent in extracts from frog skins including those from dendrobatid frogs (J. Daly and J. Waters, unpublished observations). For example, the skin of a large dendrobatid frog such as *D. auratus* has a wet weight of 250–300 mg and contains slightly more than 1 mg cholesterol. Cholesterol has also been isolated from liver and muscle of frogs[9] and both cholesterol and cholesterol acetate were isolated from frog ovaries.[10] Esterified and free cholesterol have been reported in a variety of organs, in blood and in skin of toads.[11–13] The ratio of free to esterified cholesterol in toads decreases in brain, heart, muscle and skin in the active season and increases during the dormant season.[11] The reverse occurs in lung, spleen, kidney and adrenals. The present studies demonstrate that radioactive cholesterol and mevalonate administered interperitoneally to dendrobatid frogs are incorporated to a surprisingly large extent into cholesterol and cholesterol acetate in the skin. Radioactive acetate is also incorporated to a significant extent into these skin sterols. The ratio of radioactive cholesterol to cholesterol acetate is near unity in *P. aurotaenia*, while in the two species of *Dendrobates*, radioactive cholesterol is predominant (Table 2).

Parallel experiments with *P. aurotaenia* with *dl*-serine-3-^{14}C over a period of only 2 weeks did not result in measurable incorporation of radioactivity into the 7-membered 14β-18β-heterocyclic ring of batrachotoxin (D. F. Johnson and J. W. Daly, unpublished results). Neither pregnenalone-4-^{14}C nor progesterone-4-^{14}C was converted to batrachotoxin over a period of 6 weeks of injections with *P. aurotaenia*, nor were they concentrated to a measurable extent in skin during this period (D. F. Johnson and J. W. Daly, unpublished results).

Acknowledgements—The authors express their indebtedness to Dr. Charles W. Myers, American Museum of Natural History, New York, N.Y., for the frogs used in the present experiments. They also thank Mrs. Louise Atwell, who has maintained the frog colonies at NIH.

REFERENCES

1. T. Tokuyama, J. W. Daly and B. Witkop, *J. Am. chem. Soc.* **91,** 3931 (1969).
2. E. X. Alburquerque, J. Daly and B. Witkop, *Science* **72,** 995 (1971).
3. J. W. Daly, T. Tokuyama, C. Habermehl, I. L. Karle and B. Witkop, *Justus Liebigs Annln Chem.* **729,** 198 (1969).
4. E. C. Gross and A. M. Porto, *An. Asoc. quim. argent.* **55,** 177 (1967).
5. A. M. Porto and E. G. Gros, *Experientia* **26,** 11 (1970).
6. C. Chiadao and M. V. Osuch, *Biochem. Pharmac.* **18,** 1797 (1969).
7. M. D. Siperstein, A. W. Murray and E. Titus, *Archs Biochem. Biophys.* **67,** 154 (1957).
8. C. Habermehl and A. Haaf, *Chem. Ber.* **101,** 198 (1968).
9. H. Anciaux, *Verh. K. vlaam. Acad. Wet. Klasse* **14,** 5 (1952).
10. E. M. Boyd, *J. Physiol., Lond.* **91,** 394 (1938).
11. K. Sato, *Scient. Pap. Coll. gen. Educ. Tokyo* **1,** 73 (1951).
12. R. Hüttel and H. Behringer, *Hoppe-Seyler's Z. physiol. Chem.* **245,** 175 (1937).
13. F. J. Costa de Aguiar, *Anais Fac. Med. Univ. Recife* **17,** 123 (1957).