Biochemical Pharmacology, Vol. 18, pp. 1803–1808. Pergamon Press. 1969. Printed in Great Britain

FATE OF EXOGENOUS MARINOBUFAGIN-^{3}H IN *BUFO MARINUS**

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(*Received* 25 *June* 1968; *accepted* 9 *December* 1968)

Abstract—After marinobufagin-^{3}H was injected into toads, 80 per cent of the radioactivity could be extracted from various tissues 2–3 months later. One-half of the tissue radioactivity was found in the ovarian tissues, of which 80–90 per cent could be identified as unchanged marinobufagin by thin-layer chromatography and reverse isotope dilution techniques. Of the other tissues, 27·2 per cent was found in the gastrointestinal tract. Skin and parotoid glands, where these steroids presumably are synthesized and stored, had together only 1 per cent activity. Other tissues had a total of less than 0·25 per cent of the injected radioactivity. Little was excreted.

The major conversion product of marinobufagin is tentatively identified as telocinobufagin. Thus the formation of 14β-hydroxyl group of cardiotonic steroids, at least in toads, would be by reduction of a 14β, 15β-epoxide.

IN SPITE of the ancient and widespread medicinal usage of cardiotonic steroids from the skin, venom and parotoid glands of toads, the bufadienolides,[1] little is known of their physiological role in these animals. Nor is there any information concerning their metabolic fate and tissue distribution. They are presumed to be synthesized and stored in the parotoid glands.[2] To investigate some of these problems, tritium-labeled marinobufagin was prepared and administered to *Bufo marinus* toads and its metabolism, distribution in the body and excretion were studied. Marinobufagin was chosen over other bufadienolides because of its relative abundance in the venom of this species of toad[3] and because of the key position it occupies in the biosynthetic pathway of this group of steroids.

MATERIALS AND METHODS

Toads. The toads used in these experiments were female *Bufo marinus* weighing 170–200 g, supplied by Tarpon Zoo of Tarpon Springs, Fl., claimed to be from the coastal lowlands of Columbia, South America. We were unable to determine the sex of these toads with certainty from their outside appearance, although in general the females are larger and contain more venom in their parotoids. Most of the animals we used proved to be female.

During the experiments, the toads were kept at room temperature (22–24°) in deep plastic pans containing a little water and covered loosely with a glass plate. They were fed mealworms.

* The work was supported by National Science Foundation Grant No. G-23382.
† Supported by U.S. Public Health Service, National Institutes of Health Predoctoral Training Grant No. 5 TO1 GM00943 awarded to the Department.

Isotopically labeled steroids. Radiopure cholesterol-4-^{14}C, sp. act., 55·5 μc/mg, was obtained from New England Nuclear Corp., Boston, Mass., and used without delay and further purification.

Marinobufagin was isolated from dried, Jamaican *Bufo marinus* venom by acetone extraction, column chromatography and repeated recrystallization, m.p. 218–220·5° (Kofler block, uncorrected) and gave no melting point depression when mixed with an authentic sample of marinobufagin (m.p., 224–225°[8]). This material was sent to Nuclear Research Chemicals, Inc., Orlando, Fl., for random tritiation by Wilzbach's method.

Portions of the crude tritiated marinobufagin were purified twice by preparative thin-layer chromatography. An aliquot of the purified material was chromatographed on a thin-layer plate against reference marinobufagin. Radioscan on Vanguard autoscanner model 880, Glass Plate scanner model 885, indicated one radioactive peak at the same position as the reference.

Experimentation. The parotoid glands of the toads were squeezed with the fingers immediately before injection. Cholesterol-4-^{14}C was dissolved in propylene glycol and injected subcutaneously in the inguinal region into two groups of three toads. One group of three toads was injected subcutaneously (s.c.) in the inguinal region and another group of three toads was injected under the right parotoid gland with marinobufagin-^{3}H in propylene glycol. Injection under one parotoid gland stimulated venom secretion from both glands during the injection and for about 1 hr thereafter. This secreted venom was collected by wiping with tissue, pooled, extracted with acetone and counted for tritium to ascertain the actual radioactivity that remained in the toads.

For the first 24 hr after injection, the toads were kept in dry tanks. At the end of this period the tanks were washed thoroughly with water and the wash extracted with methylene chloride. Aliquots of extracts were counted for radioactivity. Water was then added to the tanks, changed periodically, collected and pooled during the first 2 weeks. An aliquot of pooled material was solubilized with Nuclear-Chicago solubilizer and counted. The rest of the water wash was extracted with methylene chloride and aliquots counted.

At 1, 2 and 4 weeks after injection the venom was collected by squeezing the parotoid glands and wiping with pre-extracted laboratory tissue. Venom from each group of three toads was pooled and extracted with acetone in a Soxhlet extractor. One aliquot was counted directly for radioactivity. A second aliquot was subjected to two-dimensional thin-layer chromatography with ethyl acetate as solvent for both dimensions, against and with reference bufadienolides. The bufadienolides thus separated were located as dark spots against a lighter background under short-wave ultraviolet light (max., 253·7 mμ). The spots were scraped off, eluted with chloroform–methanol (9:1) and counted in a Packard Tri-carb liquid scintillation counter, model 314 EX. All radioactivity measurements were done in triplicate, corrected for background with an elution of an equivalent TLC plate area and corrected for changes in counting efficiency during the course of the experiments.

The distribution of tritium in the tissues was studied in one toad at 64 days and a second at 92 days after the injection of marinobufagin-^{3}H under the parotoid gland. The animals were anesthetized with ether, sacrificed and the organs immediately removed and frozen. Each organ or tissue examined was cut into small pieces and

homogenized in a blendor with frequent changes of isopropanol. Each extract was filtered and concentrated *in vacuo* (15 ± 4 mm Hg). Aliquots were counted to determine the total radioactivity in each extract.

Thin-layer chromatography (TLC) was always done with alumina plates. Any special procedures are included with the results.

The major bufadienolides peculiar to *Bufo marinus* are resibufogenin, bufalin, marinobufagin and telocinobufagin.

RESULTS AND DISCUSSION

Venom. It is generally assumed, though without any direct evidence, that bufadienolides are synthesized and stored in the parotoid glands.[2] Thus, it seemed preferable that the radioactive material be injected at this site. However, this was not possible, as the gland is made of collagenous material that is difficult to penetrate with a hypodermic needle. In addition, attempts to inject at this site stimulated prodigious secretion of venom. When the injection was done immediately under the bone-hard gland, it did not greatly affect the radioactivity that appeared in the venom. It did, however, stimulate the secretion of large amounts of venom for 1 hr afterward; this venom contained 8·9 per cent of the injected radioactivity. As the skin of toads, especially over the back, is leathery, needle holes reseal poorly. One is never certain, when wiping the parotoid area with tissue, whether some of the injected material was indeed from the venom by way of absorption into the gland or due to leakage through the needle hole.

Cholesterol-4-^{14}C was studied to compare the degree of incorporation of radioactivity into the venom components in the toads kept under the same conditions as those given marinobufagin-^{3}H. The incorporation of cholesterol-4-^{14}C was small with only 0·45 per cent of injected radioactivity appearing in the venom in a 4-week period. Furthermore, the radioactivity extractable with aceton was primarily associated with material less polar than bufadienolides, probably largely unchanged cholesterol. Marinobufagin and telocinobufagin, the only two bufadienolides found, together amount to only 2·7 per cent of the acetone extract, or 0·012 per cent of the total injected activity. Earlier experiments gave similar results.[4] A 2 per cent conversion has been reported[5] for marinobufagin extracted from both parotoid glands of toads given labeled cholesterol.

The venom collected during the first 4 weeks from toads injected with marinobufagin-^{3}H in the inguinal region contained merely 0·11 per cent of injected radioactivity, of which 64·2 per cent was unchanged marinobufagin and 2·2 per cent, telocinobufagin. The parotoid-injected animals contain 0·14 per cent of the total of which 62·5 per cent was marinobufagin and 8·2 per cent, telocinobufagin. The site of injection, thus, did not alter the composition of the venom: marinobufagin and telocinobufagin were clearly recovered in every sample. Small amounts of radioactivity that migrated with resibufogenin and bufalin were also found in the venom extract.

To identify these bufadienolides-^{3}H, the remaining extract of venom obtained in the first week from parotoid-injected animals was acetylated with acetic anhydride and pyridine. A portion of the acetylated extract was subjected to TLC, developed with chloroform in the first direction and ethyl-acetate in the second. There was significant retention of activity in all four bufadienolide areas, which further supports their identity. It does not seem likely that marinobufagin would be metabolized to

resibufogenin or bufalin. More likely, marinobufagin would be metabolized to some other compounds that have similar R_f values as these two bufadienolides on thin-layer plates. A lack of sufficient reference compound prevented further investigation of this material.

For further identification, the radioactive telocinobufagin eluted from the thin-layer plate was combined with 5·64 mg of authentic telocinobufagin and chromatographed on a preparative thin-layer plate. Elution and recrystallization from a mixture of ethyl–acetate and hexane yielded a material of constant specific activity with an absorption peak of 270 mμ, which was different from the bufadienolide maximum of 300 mμ. Further repeated thin-layer chromatography did not change the absorption maximum of 270 mμ. Apparently, chemical reaction(s) took place during the process of purification. Still, the observation that the radioactivity remained with the originally authentic telocinobufagin offers additional proof of its identity.

Excreta. Cholesterol-4-^{14}C-injected toads excreted less than 0·2 per cent of the radiolabel over the 2-week period following injection. The group of toads injected in the inguinal region with marinobufagin-^{3}H excreted 6·4 per cent of the injected radioactivity, while those injected under the parotoid gland excreted 11·0 per cent. The larger excretion of radioactivity after injection under the parotoid gland may be due to leakage of radioactive material from the injection site which adhered to the skin and slowly desquamated.

TABLE 1. TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER A SINGLE INJECTION OF 7·8 μc OF MARINOBUFAGIN-^{3}H UNDER THE PAROTOID GLAND*

Tissue	Per cent recovered from one toad sacrificed at:	
	64 days	92 days
Gonadal tissue	49·4	52·71
Ova-ovaries		52·5
Oviduct		0·028
Fat bodies		0·131
Fat		0·055
Venom	0·14	
Parotoid glands	0·084	
Skin	0·96	
Kidneys and adrenals	0·020	
Heart	0·058	
Gastrointestinal tract	27·2	
Other organs	0·082	
Carcass	0·023	
Total body recovery	78·19	

* This does not include the radioactivity recovered in the venom and in the excreta during the 2 weeks subsequent to injection.

*Tissue distribution. Iso*propanol extracts of tissues of a toad given marinobufagin-^{3}H under the parotoid and sacrificed on the 64th day after injection contained a total of 78 per cent of the label injected (Table 1). The largest amount of tritium was found in gonadal tissue and associated fat particles (49·4 per cent). The gastrointestinal tract had considerable radioactivity, 27·2 per cent of the injected label. The skin had 0·96 per cent, muscle and heart only 0·23 and 0·06 per cent, respectively.

Just before sacrifice both parotoid glands were squeezed free of venom. The venom

(50 days after last squeezing) extract contained only 0·14 per cent, whereas the parotoid extract had even less, 0·084 per cent, of the radioactivity injected.

The gonadal tissue of a second toad sacrificed at 92 days was separated into ovarian tissue, oviduct and fat bodies. The isopropanol extracts of the oviduct and of the fat bodies had little radioactivity. The ovarian tissue had 52·5 per cent of the injected radioactivity.

An aliquot, representing 20 per cent of the ovarian tissue extract of the 92-day toad ($2{\cdot}89 \times 10^5$ cpm), was concentrated to an oil. This was hydrolyzed in methanol–ether solution with an excess of aqueous 2N K_2CO_3 at room temperature for 24 hr. The hydrolysate was then extracted with ether and washed with water until neutral. There was little or no radioactivity in the water extract. After the removal of ether, the residue was chromatographed on a florisil column. The bufadienolides and radioactivity appeared in the fractions beginning with 1% acetone in ether. These were combined to yield 145 mg of oil. This resulting oil was then chromatographed on a Woelm neutral alumina column, eluting with ether containing increasing amounts of acetone. Almost all of the radioactivity recovered (88·5 per cent) was found in the first fraction with 100% acetone. This fraction had only one ultraviolet light absorbing area on an alumina thin-layer plate with a R_f value corresponding to the reference marinobufagin. Elution of this spot gave 74 per cent of the plated radioactivity.

One-half of the material obtained from Woelm alumina chromatography was further purified by preparative alumina thin-layer chromatography yielding 3·14 mg marinobufagin and 77,600 cpm. To this was added 10·06 mg authentic unlabeled marinobufagin and the mixture was recrystallized three times from aqueous methanol. After each recrystallization the material was dried, weighed and aliquots counted to determine its specific activities. Along with the weight, u.v. absorption at 300 mμ was made to further ascertain its purity. On the basis of specific activity after the first recrystallization, 92 per cent of it could be accounted for as marinobufagin.

The remainder of the original 92-day toad gonadal extract was acetylated and a portion of this crude acetate was subjected to two-dimensional TLC. Only one ultraviolet light absorbing spot was detected and this had an R_f value corresponding to authentic acetyl marinobufagin. The extract of this spot contained 87 per cent of the plated radioactivity. As the recovery from thin-layer plates was generally between 85–95 per cent in our laboratory, 87 per cent recovery here indicated that all, or nearly all, of the radioactivity was associated with marinobufagin acetate. Furthermore, some loss is expected in the acetylation step.

One of the reasons for this study stemmed from our observation[4] that the conversion of labeled-steroid precursors, cholesterol and bile acids, to venom bufadienolides was surprisingly low. The label seemed to stay in the body without appearing in the excreta in any appreciable quantities. This could be due to tissue binding or an extensive metabolism to carbon dioxide and water. Since the steroid nucleus withstands catabolism very well in other animals, the tissue-binding hypothesis seemed more likely. Preliminary studies of tissue binding when a labeled bile acid was injected into toads had indicated that neutral radioactive material remained mostly in the viscera other than the heart. Therefore, it was decided to study the distribution of labeled marinobufagin, hoping that it might provide information not only on tissue concentration but also on its metabolism and possibly its biological importance to the toads.

The low conversion of cholesterol to bufadienolides was clearly shown in the venom.

That some marinobufagin-^{3}H readily enters the parotoid allows some doubt as to whether this is the locus of synthesis as generally assumed. The small amounts of bufadienolides found in the parotoid do not necessarily mean that the gland is the site of their origin.

Briefly restated, the results showed that the bulk of marinobufagin-^{3}H given to female toads remained unchanged in the gonadal tissue for an extended period of time. The only metabolite appeared to be, though yet uncertain, venom telocinobufagin. One may reasonably postulate that the conversion to telocinobufagin likely involved a reduction of the epoxide group in marinobufagin. Since epoxidation can only be brought about on a double-bonded molecule, the epoxide group in marinobufagin would be from a $^{14}\Delta$-steroid.

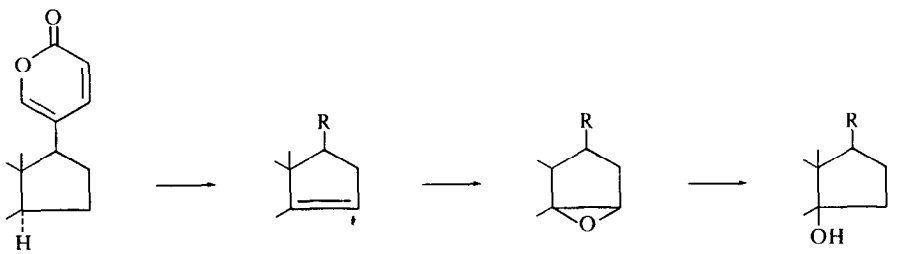

FIG. 1. Biosynthetic scheme.

Verification of this suggestion may be technically feasible if some of the suspected intermediates can be synthesized.

Acknowledgements—We wish to thank Prof. P. C. Feng, Kingston, Jamaica, for the gift of dried venom, and Prof. Kuno Meyer, Basel, Switzerland, for the gift of four samples of reference toad bufadienolides.

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