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BIOSYNTHESIS OF BUFADIENOLIDES—3β-HYDROXYCHOLANATES AS PRECURSORS IN *BUFO MARINUS* BUFADIENOLIDES SYNTHESIS*

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Abstract—The biosynthesis of bufadienolides in toads (*Bufo marinus*) was investigated by measuring the radioactivity of resibufogenin, bufalin, marinobufagin and telocinobufagin in the venom following injection of some possible precursors labeled with ^{14}C or tritium. The data indicate that both 5α and 5β, 3β-hydroxycholanate derivates are more efficient precursors than cholesterol. No incorporation could be detected from the venom extract when labeled pregnenolone was given.

These findings are in direct contradistinction to the reported biosynthesis of cardiotonic steroids in plants from pregnenolone or progesterone.

SIPERSTEIN *et al.*[1] have shown that cholesterol-4-^{14}C is a precursor of a bufadienolide, marinobufagin, in the parotoid glands of *Bufo marinus*, while Tschesche and Prassat[2] have shown that, in the plant species *Helleborus atrorubens*, 5-pregnen-3β-ol-20-one-21-^{14}C-glycoside was utilized to form the bufadienolide hellebrin.

Cholesterol is metabolized in animals to both pregnenolone and hydroxycholanates, the bile acids. It seems possible that a bile acid which would contain all 24 carbon atoms could be a possible intermediate in the synthesis of bufadienolide from cholesterol.

In order to test this postulate, some possible steroid precursors labeled with ^{14}C or ^{3}H were administered subcutaneously to *Bufo marinus* toads and the exuded venom was assayed for incorporation of radioactivity into the bufadienolides. The steroids injected were cholesterol-4-^{14}C, pregnenolone-4-^{14}C, methyl 3β-formoxy-5β-cholanate-24-^{14}C, sodium 3β-hydroxy-5β-cholanate-24-^{14}C, methyl 3β-acetoxy-5-cholenate-^{3}H, methyl 3β-hydroxy-5α-cholanate-^{3}H and methyl 3α-hydroxy-5β-cholanate-24-^{14}C.

MATERIALS AND METHODS

The toads used in these experiments were female *Bufo marinus*, obtained‡ in Columbia, South America, weighing 170–200 g at the beginning of the experiments. They did not gain appreciably in weight during the 3 months they were kept in the laboratory. These toads were kept at room temperature (22–24°) in deep plastic

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‡ Purchased from Tarpon Zoo, Tarpon Springs, Fl.

pans containing a little water and covered loosely with a heavyglass plate. Mealworms and angleworms constituted their sole source of food.

All steroid compounds studied were dissolved in propylene glycol, except the sodium salt of 3β-hydroxy-5β-cholanate-24-^{14}C which was dissolved in water. Three animals were employed in each group. All toads were injected subcutaneously (s.c.) in the inguinal region. Immediately after injection the parotoid glands were squeezed with fingers to express the existing venom in order to stimulate the production of new venom sacs and their contents.[3]

At 1, 2, 4 and 6 weeks after injection, the venom from each group of three toads was collected, pooled and immediately extracted with acetone in a Soxhlet extractor. From each extract, aliquots were removed and counted for radioactivity; the remainder was chromatographed on a thin-layer alumina plate, developed with chloroform in one direction and ethyl acetate in the other direction. This two-dimensional chromatography gives good separation of the bufadienolides in well defined spots. After location of the bufadienolides on the plates by ultraviolet light (max., 253·7 mμ),* the spots were scraped off and extracted with chloroform–methanol (9:1) mixture. Aliquots of these extracts were employed to measure radioactivity in a Packard Tri-carb, model 312 EX counter. Equivalent areas of the TLC plate were extracted and counted for the background correction.

Cholesterol-4-^{14}C. Radiopure cholesterol-4-^{14}C was obtained from New England Nuclear (sp. act., 55·5 μc per mg) and used without further purification. Three toads received 4·63 × 10^6 cpm each or a total of 13·9 × 10^6 cpm (11·0 μc).

Pregnenolone-4-^{14}C. Radiopure pregnenolone-4-^{14}C was obtained from Nuclear-Chicago Corp. (sp. act., 76·0 μc per mg) and used without further purification. Three toads received 3·0 × 10^6 cpm each or 9·02 × 10^6 cpm total (7·2 μc).

Methyl lithocholate-24-^{14}C. Lithocholic acid-24-^{14}C (3α-hydroxy-5β-cholanic acid) was obtained from Tracerlab-Keleket (sp. act., 8·00 μc per mg). The methyl ester was prepared in methanol–ether solution with distilled diazomethane and purified by preparative alumina thin-layer chromatography. Three animals received 3·9 × 10^6 cpm each or 11·7 × 10^6 cpm total (9·4 μc).

Methyl 3β-formoxy-5β-cholanate-24-^{14}C. Methyl 3β-formoxy-cholanate-24-^{14}C was prepared from lithocholic acid-24-^{14}C by the method of Chang and Blickenstaff[4] with slight modifications. Methyl 3α-tosyloxycholanate-24-^{14}C, synthesized at room temperature and partially purified by Woelm-alumina column chromatography, was epimerized in dimethyl–formamide at 70–73° for 24 hr to methyl 3β-formoxy-5β-cholanate-24-^{14}C. The material was chromatographed twice on florisil columns. The crystalline material obtained had the same thin-layer chromatographic mobility as the authentic compound and gave a single radioactive peak.

Three toads received 1·26 × 10^6 cpm each of 3·78 × 10^6 cpm total (3·02 μc).

Sodium 3β-hydroxy-5β-cholanate-24-^{14}C. The sodium salt was prepared by sodium hydroxide hydrolysis of methyl 3β-formoxy-5β-cholanate-24-^{14}C in methanol solution at room temperature.

Each of 5 toads received 1·5 × 10^5 cpm in 0·5-ml aqueous solution or 7·5 × 10^5 cpm total (0·60 μc).

Methyl 3β-acetoxy-5-cholenate-^{3}H. 3β-Hydroxy-5-cholenic acid was randomly

* Occasionally, fluorescent plates, which markedly improve visualization under ultraviolet light irradiation, were used.

labeled with tritium by the Wilzbach technique and the labile tritium removed by solvolysis by Tracerlab-Keleket. The methyl ester was prepared with diazomethane in ether and the acetate with acetic anhydride and pyridine at room temperature. After extraction with ether and removal of excess reagents, the ether solution was concentrated to dryness. The product was recrystallized once from acetone and chromatographed through a Woelm alumina column. Methyl 3β-acetoxy-5-cholenate-^{3}H thus obtained was further recrystallized from hexane and then from acetone to give a material melting at 157·5 to 158° (Kofler block, uncorrected) identical to the authentic compound of 155·5 to 158° (m.p. given 155°[5]). Thin-layer chromatography yielded a single radioactive peak.

Propylene glycol solution of this steroid was given to three toads, each receiving $3{\cdot}87 \times 10^{6}$ cpm or a total of $11{\cdot}6 \times 10^{6}$ cpm (31 μc).

Methyl 3β-hydroxy-5α-cholanate-^{3}H. An ethanolic solution of 3β-hydroxy-5-cholenic acid was hydrogenated over palladium-on-charcoal at room temperature and atmospheric pressure. The product, after purification, melted at 217–218° (217–218°[6]). This material was randomly labeled with tritium by the Wilzbach technique by Tracerlab-Keleket. The methyl ester was made with diazomethane. Purification of this material gave an ester of m.p. 150·5–151°, (151–153°[6]). This material gave a single radioactive peak on a thin-layer chromatographic plate.

Each toad received $6{\cdot}27 \times 10^{6}$ cpm or a total of $18{\cdot}8 \times 10^{6}$ cpm for three toads (50 μc).

Reference bufadienolides. Authentic crystalline resibufogenin, bufalin, marinobufagin and telocinobufagin were gifts from Professor Kuno Meyer* of Basel, Switzerland.

RESULTS AND DISCUSSION

In addition to a number of preliminary experiments designed to ascertain the technical feasibility and theoretical soundness of our proposal, each experiment was repeated at least two times, though only one set of data is presented here, representing results from one group of three toads. Furthermore, other similar steroids were studied. These included progesterone-4-^{14}C, pregnenolone-16-^{3}H, pregnenolone-7-^{3}H, methyl 3β-hydroxy-5β-cholanate-^{3}H and methyl 3β-hydroxy-5β-cholanate-24-^{14}C. Data on these other experiments are not reported here because they were done on different shipments of toads, during a different season of the year, or there was insufficient material for definitive results. The results are, however, in general agreement with those reported; namely, all bile acids with a 3β-hydroxy group were precursors whereas none of the other steroids were incorporated.

The experiments presented here were done on one shipment of adult female toads of about the same size and handled the same way to minimize variables. They were kept at room temperature in the same region of the laboratory and received the same amount of food. Each group of three animals was kept in one pan. Collection of venom was made at the same time intervals and always at the same time of the day. The venom from a group of three toads was pooled to assure sufficient material for assays and this also tended to minimize individual differences.

The degree of synthesis based on tritium incorporation of randomly labeled

* The authors wish to thank Prof. Meyer for the generous gift.

steroids in the experiments can be considered as a minimum level due to the inevitable loss of tritium during the biosynthetic processes.

The incorporation of radioactivity into the bufadienolides in the venom is small, with a maximum of 0·5 per cent in 6 weeks time, when calculated on the basis of total radioactivity injected. However, of the total radioactivity that reached the parotoid glands and was exuded in the venom as acetone extractable components, as high as 66 per cent could be accounted for as bufadienolides by thin-layer chromatography.

The amount of venom obtainable varies with individual toads, but the relative abundance of venom bufadienolides did not vary greatly, and was in general agreement with values reported for dried venom of *Bufo marinus* which contained 10 per cent marinobufagin, 0·8 per cent telocinobufagin, 0·7 per cent bufalin and 0·07 per cent resibufogenin.[7]

Cholesterol was a rather poor precursor (Table 1). There was a barely detectable amount of marinobufagin-^{14}C in the venom during the 6 weeks after the administration of cholesterol-4-^{14}C. Many attempts to improve the yield during a 2-year span were unsuccessful. Though the yields were small, some radioactive marinobufagin was

TABLE 1. CONVERSION OF LABELED STEROIDS INTO TOAD VENOM BUFADIENOLIDES*

	Cholesterol-4-^{14}C (cpm)	Pregnenolone-4-^{14}C (cpm)	Me-3α-hydroxy-5β-cholanate-24-^{14}C (cpm)	Me-3β-formoxy-5β-cholanate-24-^{14}C (cpm)	Na-3β-hydroxy-5β-cholanate-24-^{14}C (cpm)	Me-3β-acetoxy-5-cholanate-^{3}H (cpm)	Me-3β-hydroxy-5α-cholanate-^{3}H (cpm)
First week:							
Total acetone-extractable	1600	400	2000	5000	3000	4000	700
Resibufogenin	0	0	0	0	2100	0	0
Second week:							
Total acetone-extractable	9400	400	7000	3000	95,000	4000	600
Resibufogenin	0	0	0	0	47,000	0	0
Fourth week:							
Total acetone-extractable	1000	200	100	600	33,000	19,000	10,000
Resibufogenin	0	0	0	0	0	2800	300
Bufalin	0	0	0	0	0	4200	3300
Marinobufagin	130	0	0	0	13,000	1300	500
Telocinobufagin	0	0	0	0	0	0	300
Sixth week:							
Total acetone-extractable	2600	100	200	2000	5000	2600	1800
Resibufogenin	0	0	0	850	0	400	200
Marinobufagin	260	0	0	0	0	0	400
Telocinobufagin	0	0	0	0	0	0	400
%Bufadienolides of Total acetone-extractable	2·7%	0%	0%	8%	66%	30%	46%

* The total radioactivity recovered in the acetone extracts of venom samples and that portion of radioactivity in the resibufogenin, bufalin, marinobufagin, or telocinobufagin spots on thin-layer chromatograms are indicated. All radioactivity measured is normalized on the basis of 1×10^7 cpm injected doses.

always present. Siperstein *et al.*[1] reported that 2 per cent of cholesterol was converted to marinobufagin in both glands in 72 days while we could obtain only 0·004 per cent in the venom in 42 days and 0·009 per cent in 71 days. The reason for such large discrepancy is unknown.

When toads were injected with pregnenolone-4-^{14}C, the radioactivity in the venom was barely detectable. With the injection of methyl 3α-hydroxy-5β-cholanate-24-^{14}C (methyl lithocholate) somewhat greater amounts of radioactivity were found in the venom extracts. In neither case was any radioactive bufadienolide found by the thin-layer chromatographic method of identification. Also 3α-hydroxy-5β-cholanic acid did not appear to be a precursor for bufadienolides. However, 3β-hydroxy bile acids, esterified or free, were utilized in bufadienolide synthesis. Equally interesting was the incorporation of a 5α-cholanic acid.

From the per cent conversion, it seems clear that the whole side-chain of 3β-isomers was used in the synthesis without being cleaved to a pregnane derivative. Should the side-chain be degraded into 2 or 3 carbon units and enter into the general metabolism, one would expect much lower incorporation than the 66 per cent found in total venom activity. Proof of utilization of the intact bile acid side-chain would require evidence that C-24 in the synthesized bufadienolides be labeled. This would require considerably more material than is available at present.

Because of the limitations of the system, nothing definite can be said about the

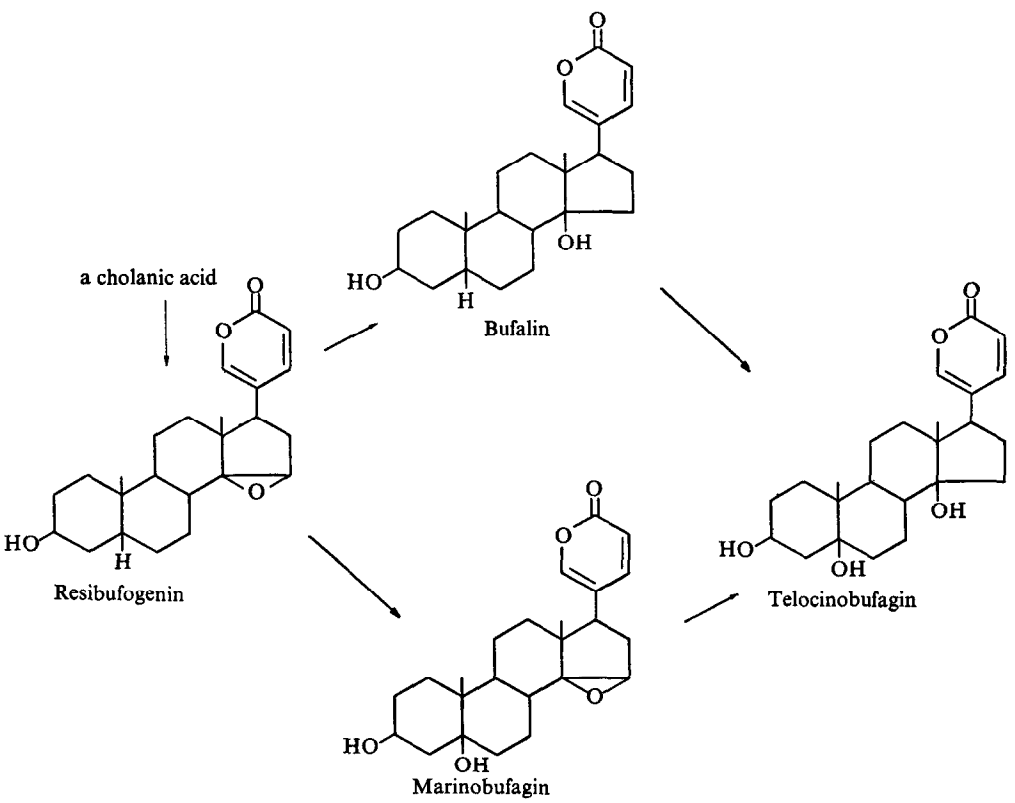

FIG. 1. Biosynthetic scheme.

order or preference for 5- and 14-hydroxylation and 14, 15-epoxidation except that 14 or 14,15-oxygenation precedes 5-hydroxylation. It seems only obvious as no bufadienolide with a 5-hydroxy group but without an oxygen function in the 14-15 position is known. Nothing at all can be said about whether or not the lactone is formed first before any nucleus oxygenation can take place. From the scanty information available, it seemed that resibufogenin was the first to appear, as in four venom extracts it was the sole product. On the two occasions when bufalin was found it contained the most radioactivity, which is particularly interesting since its natural abundance is only 7 per cent of marinobufagin. Thus, it seems reasonable to postulate that resibufogenin is first formed and metabolized by a process either of reduction to bufalin or of further hydroxylation to marinobufagin. By the same processes of reduction or hydroxylation, marinobufagin and bufalin are, respectively metabolized to telocinobufagin. Further information would be needed for detailed pathway.

It is curious that methyl 3β-hydroxy-5α-cholanate is an efficient substrate. All four of the bufadienolides studied here are 5β-isomers. Inversion without a concomitant reaction is unknown in biochemistry and is mechanistically difficult to explain. It is possible that the radioactive bufadienolides formed with the 5α-precursor were 5α-isomers. Our only criterion in identifying these steroids was by their mobility on thin-layer chromatography compared with authentic compounds. This is suitable with naturally occurring bufadienolides extracted from venoms, but may not be adequate in differentiating the 5α-isomers from 5β-isomers.

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