Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 11–17. Pergamon Press. Printed in Great Britain

BIOSYNTHESIS OF BUFADIENOLIDES IN TOADS

III*—EXPERIMENTS WITH [2-^{14}C]MEVALONIC ACID, [20-^{14}C]3β-HYDROXY-5-PREGNEN-20-ONE AND [20-^{14}C]CHOLESTEROL

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(*Received* 30 *July* 1971)

SUMMARY

The biosynthesis of bufadienolides in *Bufo paracnemis* toads has been studied in different seasons of the year using [2-^{14}C]mevalonic acid, [20-^{14}C]pregnenolone, and [20-^{14}C]cholesterol as precursors. The results showed that the doubly unsaturated δ-lactone ring of the bufadienolides is directly derived from the cholesterol side chain without the compound's prior conversion into pregnenolone. The probable sequence in which the different bufadienolides are formed is discussed.

THE BIOSYNTHESIS of sterols in plants is assumed to follow a pattern analogous to that in animals[2–4]. This assumption is supported by the demonstration of the biosynthesis of cholesterol from mevalonic acid in *Digitalis purpurea*[5], by the transformation of cholesterol into pregnenolone[6] and by the conversion of the latter into cardenolides[7, 8] and bufadienolides[9, 10] in plants. It has also been shown that pregnenolone is not converted into bufadienolides in animals[10] but that bile acids[11] and cholesterol[12, 13] are good precursors of bufadienolides in the toads *Bufo marinus* and *Bufo paracnemis*.

In one experiment[13], [20-^{14}C]cholesterol[14] was injected into several toads, and the bufadienolide marinobufagin (VIII) was isolated after different periods of time. Its radioactivity confirmed the precursor role of cholesterol. Degradation of the bufadienolide demonstrated that there was no randomization of activity and that the label was located at the expected position.

In our first paper in this series[15] it was shown that, strangely enough, [2-^{14}C]mevalonic acid seems to be a poor precursor of animal bufadienolides since 18 days after its administration the isolated bufadienolides were almost devoid of activity. It was later suspected that the failure of the animal to incorporate mevalonic acid into the bufadienolides could be attributed not to the lack of an enzymic system able to use mevalonic acid for the formation of the steroid nucleus, since cholesterol in liver and the so-called γ-sitosterol in parotid glands were fairly radioactive, but to the short duration of the experiment.

Consequently, we injected [2-^{14}C]mevalonic acid into several intact specimens of *B. paracnemis* toads but on this occasion the venom from the parotid and tibial glands was collected much later than in the earlier experiment. The bufadienolides were isolated as previously described[15, 16] and their radioactivities determined.

*For Part II, see Ref. [1].

A similar experiment was carried out in winter in order to establish whether hibernation affects the biosynthesis of bufadienolides.

In addition, the metabolism of [20-^{14}C]cholesterol and [20-^{14}C]pregnenolone [17] was investigated in hibernating animals.

EXPERIMENTAL

General

Melting points were determined with a Fisher-Johns apparatus and are uncorrected. Preparative t.l.c. was conducted on aluminun oxide (Merck. PF_{254}, type E). Rotations were taken at 22°C in chloroform solution. Radioactivity was counted with a Packard Tri-Carb model 93320 liquid scintillation spectrometer in the usual scintillation solutions. [2-^{14}C]Mevalonic acid was purchased from New England Nuclear Corp., Mass., U.S.A. [20-^{14}C]Pregnenolone and [20-^{14}C]cholesterol were synthesized as previously described[14, 17]. Acetates were prepared with acetic anhydride : pyridine (1 : 1) following the usual procedure. *B. paracnemis* toads were collected in the surroundings of Resistencia City, province of Chaco, Argentina.

Injection of labelled precursors.

(a) [2-^{14}C]Mevalonic acid (DBED salt; 21 mg, $3{\cdot}64 \times 10^9$ dpm/mmol) was dissolved in physiological solution (10 ml) and injected subcutaneously into 10 toads as previously reported[15]; the experiment was carried out in December.

(b) The same precursor (23 mg, $1{\cdot}31 \times 10^{10}$ dpm/mmol) was given in the same manner in March to 15 toads.

(c) [20-^{14}C]Cholesterol (60 mg, $1{\cdot}93 \times 10^7$ dpm/mmol) was suspended in distilled water (28 ml) with Tween 20 (2 ml), and the suspension was injected in March into 15 toads as previously described [15].

(d) [20-^{14}C]Pregnenolone (41 mg, $7{\cdot}33 \times 10^8$ dpm/mmol) in water (23 ml) with Tween 20 (0·5 ml) was injected into 23 animals in January.

(e) The same labelled compound (40 mg, $5{\cdot}42 \times 10^7$ dpm/mmol) was emulsified in distilled water: Tween 20 (95 : 5, v/v; 15 ml) and injected into 12 toads in March.

Toads were kept indoors in glass jars over moist sand during the course of the experiments.

Isolation of sterols and bufadienolides

At times indicated in the Table, the venom from the parotid and tibial glands was collected by simple pressure; this mixture was worked up as previously described[15] leading to the isolation, by means of preparative t.l.c., of: sterol mixture (γ-sitosterol), m.p. 144–145°C, $[\alpha]_D - 38{\cdot}9°$; bufalin (V), m.p. 240–241°C, $[\alpha]_D - 9{\cdot}1°$, (acetate, m.p. 245–246°C, $[\alpha]_D - 8{\cdot}0°$); marinobufagin (VIII), m.p. 219–220°C, $[\alpha]_D + 13{\cdot}5°$, (acetate, m.p. 205–212°C, $[\alpha]_D + 28{\cdot}1°$); telocinobufagin (VI), m.p. 207–208°C, $[\alpha]_D + 8{\cdot}2°$, (acetate, m.p. 270–273°C, $[\alpha]_D + 21{\cdot}9°$). The physical constants here reported represent typical values.

Cholesterol was isolated from liver and gall bladder, and purified by previously reported methods [18].

RESULTS

All results are shown in Table 1, where activity values represent single determinations from the pooled venom of a specified number of animals. It is appropriate to point out that the experiments were started with a reasonable amount of animals. However, as it was necessary to wait for a long time after inoculation to find a measurable amount of radioactivity in the bufadienolides, and although the toads were kept in the best available conditions, some of them died, for unknown reasons, during the span of the experiment. Therefore, in some cases the determination for the longest period of time was performed with only one toad.

From the tabulated values the following observations can be made:

(a) Pregnenolone, as already reported[10], is a very poor precursor of animal bufadienolides both during activity and hibernation of the animal.

Table 1. Activities of sterols and bufadienolides obtained from [2-^{14}C]mevalonic acid, [20-^{14}C]cholesterol and [20-^{14}C]pregnenolone inoculation experiments

Precursor Specific Activity dpm/mmol	Number of animals and season of experiment	Collection time after injection (days)	Number of animals whose venom was used for radioactivity determinations	Sterol mixture		Bufalin		Marinobufagin		Telocinobufagin		Cholesterol (from liver)		Ref
				Specific activity (dpm/mmol)	Specific incorp (%)	Specific activity (dpm/mmol)	Specific incorp (%)	Specific activity (dpm/mmol)	Specific incorp (%)	Specific activity (dpm/mmol)	Specific incorp. (%)	Specific activity (dpm/mmol)	Specific incorp. (%)	
[2-^{14}C]Mevalonic acid $7{\cdot}24 \times 10^{9}$	10 summer	18	10	$3{\cdot}7 \times 10^{5}$	0·01	$3{\cdot}1 \times 10^{5}$	0·0001	$1{\cdot}2 \times 10^{3}$	0·00003	$1{\cdot}6 \times 10^{3}$	0·00004			15
[2-^{14}C]Mevalonic acid $3{\cdot}64 \times 10^{9}$	10 summer	44	4	$2{\cdot}4 \times 10^{5}$	0·006	$3{\cdot}4 \times 10^{5}$	0·009	$8{\cdot}0 \times 10^{4}$	0·002	$2{\cdot}4 \times 10^{6}$	0·066	$3{\cdot}1 \times 10^{6}$	0·09	
		114	4	$9{\cdot}1 \times 10^{5}$	0·025	*		$8{\cdot}2 \times 10^{4}$	0·002	$4{\cdot}8 \times 10^{6}$	0·13			†
[2-^{14}C]Mevalonic acid $1{\cdot}31 \times 10^{10}$	15 winter	78	4	$8{\cdot}2 \times 10^{5}$	0·006	*		$2{\cdot}6 \times 10^{4}$	0·001	$3{\cdot}7 \times 10^{4}$	0·0003			
		224	1	$5{\cdot}8 \times 10^{6}$	0·04	$1{\cdot}5 \times 10^{4}$	0·0001	$1{\cdot}4 \times 10^{4}$	0·0005	$5{\cdot}5 \times 10^{4}$	0·0004	$2{\cdot}5 \times 10^{6}$	0·02	†
[20-^{14}C] Cholesterol $1{\cdot}93 \times 10^{7}$	15 winter	78	4	$5{\cdot}0 \times 10^{4}$	0·25	$2{\cdot}6 \times 10^{4}$	0·13	$0{\cdot}6 \times 10^{4}$	0·033	$0{\cdot}7 \times 10^{4}$	0·033	$1{\cdot}9 \times 10^{4}$	0·10	‡
		107	1	$5{\cdot}8 \times 10^{4}$	0·28	*		$1{\cdot}9 \times 10^{4}$	0·10	$5{\cdot}2 \times 10^{4}$	0·27			‡
[20-^{14}C] Pregnenolone $7{\cdot}33 \times 10^{8}$	23 summer	33	5	$9{\cdot}5 \times 10^{2}$	0·0001	*		$6{\cdot}5 \times 10^{2}$	0·00009	*				§
		80	10	$2{\cdot}2 \times 10^{3}$	0·0003	*		$6{\cdot}8 \times 10^{2}$	0·00009	*				§
[20-^{14}C] Pregnenolone $5{\cdot}42 \times 10^{7}$	12 winter	146	1	$5{\cdot}3 \times 10^{2}$	0·0009	*		$2{\cdot}1 \times 10^{2}$	0·00003	$1{\cdot}5 \times 10^{2}$	0·00002			†

*Values unreliable because of insufficient amount of material.

†This paper.

‡For a preliminary communication see Ref. [13].

§For a preliminary communication see Ref. [10].

(b) Mevalonic acid was incorporated into the bufadienolides to a larger extent in summer (active life of the animal) than in winter (natural hibernation period): the same result was observed with cholesterol isolated from liver and gall bladder.

(c) The radioactivity of the sterol mixture (γ-sitosterol) and of bufadienolides in glands increased with time independent of the season.

(d) Cholesterol is a much better precursor of bufadienolides than mevalonic acid, as determined from experiments conducted at the same time of the year.

(e) When comparison was possible, bufalin (V) and telocinobufagin (VI) incorporated more radioactivity than marinobufagin (VIII) although the latter is the main bufadienolide in the mixture of venoms.

DISCUSSION

The most striking aspect of the biosynthesis of bufadienolides is that whilst plant bufadienolides, as plant cardenolides[7, 19], are derived from pregnenolone, animal bufadienolides are derived from cholesterol or a closely related compound without the formation of a pregnenolone-like intermediate. In this respect the biosynthesis of animal bufadienolides resembles that of bile acids.

The conversion of cholesterol into the bufadienolides involves a number of interesting biochemical transformations including the degradation of the side chain to a doubly unsaturated 6-membered ring lactone, the incorporation of a tertiary hydroxyl group at C-14 (bufalin, telocinobufagin, etc.) or of a 14,15-epoxide (marinobufagin, resibufagin (VII)), the incorporation of a second tertiary hydroxyl group at C-5 (marinobufagin, telocinobufagin, etc.) and the incorporation of secondary hydroxyl groups at other positions[20, 21].

None of these transformations has been studied concerning the order in which they are formed or the biochemical mechanism effecting the different steps.

The results presented here show unequivocally that cholesterol is the direct precursor of animal bufadienolides and that the pathway cholesterol → pregnenolone → bufadienolides is not operative in animals as it seems to be in plants. On the other hand, our results do not completely clarify the metabolic pathway from cholesterol to bufadienolides, although it can be assumed that, as has been demonstrated in the biosynthesis of bile acids[22], the incorporation of the oxygenated functions should occur prior to the formation of the lactone ring.

Considering two experimental results, i.e. the extremely low rate of formation of bufadienolides as compared with the formation of cholesterol in the living toad, and the fact that in the toad venom mixture no compound with a cholanic acid-type side chain has been detected, it is reasonable to assume that the slow step in the conversion cholesterol → bufadienolides should be the degradation of the side chain of cholesterol[23].

For the biological transformation of cholesterol into a bufadienolide several pathways can be postulated (see Fig. 1). Thus, one might first assume that the sequence of events is initiated by the degradation of the cholesterol side chain to a cholenic acid structure (I) because it has been demonstrated that compounds of this type were incorporated into animal bufadienolides[11]. Intermediate I could either be hydroxylated at C-14 (III) or transformed into the Δ^{14} derivative (II). The biosynthetic incorporation of a 14β-hydroxy group has been discussed and studied by several research groups[24–26] in the field of plant cardenolides, but the true biochemical mechanism has not been clarified yet; by elimination of

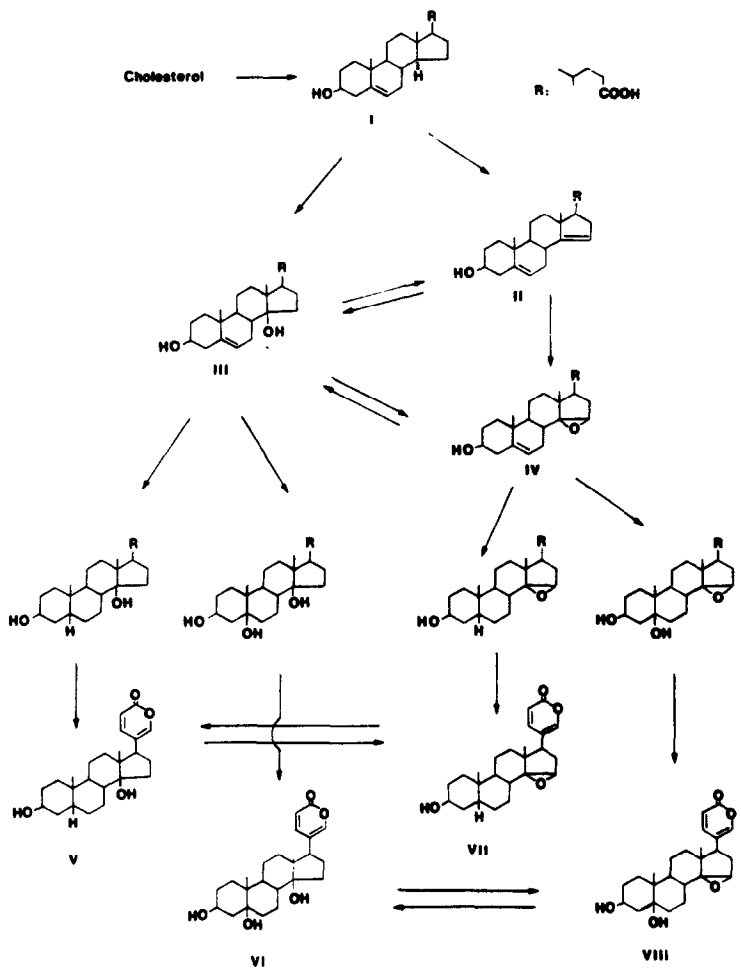

Fig. 1. Plausible biosynthetic pathways from cholesterol to bufadienolides in toads.

possibilities there remained the "free-radical process" [24] or the existence of a 14β-hydroxylase which has not been ever detected in animals nor in plants.

The formation of II from I should involve a dehydration step; the hydroxyl group could have been located at C-14 (14α) or at any other position; in the latter case isomerization of the double bond (for instance, a Δ^7) must occur.

Epoxidation of II should directly produce intermediate IV which alternatively might have been produced through the hydroxylated intermediate III. Conversion of a double bond into an epoxide via the alcohol (hydration-oxidation process) has been reported to occur in plants [27, 28].

The transformation of III and IV into the saturated (hydrogenated) derivatives would probably involve the formation of a 4-en-3-keto system as it is found in animals and also in the biosynthetic pathway from pregnenolone to cardenolides in plants [19, 29]. The introduction of a 5β-hydroxyl group would be performed by a direct hydration of the C_5–C_6 double bond since it has been demonstrated that in plants there was no hydroxylation by direct substitution of a 5β nor a 5α-hydro-

gen atom of a saturated precursor[30], and that apparently no 3-keto system was involved in the transformation.

Considering the last steps to the bufadienolides V, VI, VII and VIII, we can assume that hydroxylation at C-21 with the subsequent cyclization to the δ-lactone should be produced prior to the formation of the double bonds. Moreover, a possible conversion among bufadienolides (V ⇄ VII), (VI ⇄ VIII) cannot be discarded for the present knowledge of their biosynthesis.

Keeping in mind the several biosynthetic pathways from cholesterol to bufadienolides just outlined, our experimental result that both bufalin (V) and telocinobufagin (VI) were always more radioactive than marinobufagin (VIII) would point to the fact that the formation of bufadienolides with a 14β-hydroxyl group should precede those with a 14,15-epoxy ring. If it is assumed that intermediate III were derived from IV, bufadienolides with an epoxy ring would be formed prior to those with the 14β-hydroxyl group, but considering our results this seems not to be the case.

The problem of biosynthesis of bufadienolides, and the metabolic transformation order between the 14β-hydroxyl group and the 14β, 15β-epoxy ring require further investigation. Also, the isolation from toad venom of cardenolides having a 14β, 15β-epoxy ring[31, 32] increases the interest in this area.

ACKNOWLEDGEMENTS

We thank the Consejo Nacional de Investigaciones Cientificas y Técnicas (Buenos Aires) and FORGE (New York) for financial support.

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