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BIOSYNTHESIS OF PLANT STEROLS—III.[1,2]

MECHANISM OF SATURATION OF RING B IN PREGNENOLONE DURING ITS CONVERSION TO DIGITOXIGENIN IN *DIGITALIS LANATA*

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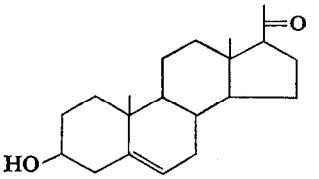
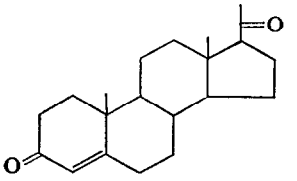
Abstract—3α-^{3}H-pregnenolone was synthesized and administered with 4-^{14}C-pregnenolone to a *Digitalis lanata* plant, from which labeled digitoxigenin was subsequently isolated. A comparison of the tritium distribution in the two compounds proved that oxidation at C_3 is an obligatory step in the biosynthetic pathway.

INTRODUCTION

THE BIOSYNTHETIC conversion of pregnenolone to the cardenolides in *Digitalis lanata* requires the reduction of the Δ^5-double bond. It was suggested that saturation of the homoallylic alcohol (Δ^5-3β-OH) in pregnenolone might proceed, as it does in animals *via* oxidation and isomerization to a Δ^4-3-ketone which is subsequently reduced.[1] Supporting evidence was provided by the conversion of pregnenolone (I) into progesterone (II) in *D. lanata* and by the incorporation of the latter into the cardenolides in the same plant.[1] After completion of this work, Graves and Smith[3] reported the conversion of progesterone to 5α-pregnane-3,20-dione (III) by cell cultures of *D. purpurea*. In addition Professor E. Heftman[4] has informed us in a private communication that he has isolated 5β-pregnane-3,20-dione from *D. lanata* after the administration of 4-^{14}C-pregnenolone.



I



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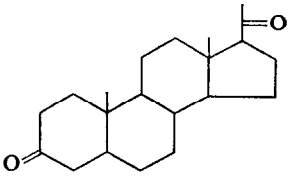

III

The fact that ring A saturated 3-ketones have been identified as metabolites of pregnenolone in *Digitalis* species does not necessarily prove that they are intermediates on the major biosynthetic route from pregnenolone to the cardenolides. Determination of the tritium content of labeled cardenolides resulting from incorporation of 3α-^{3}H-4-^{14}C-pregnenolone into *D. lanata* would decide this point and was the purpose of the present work.

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[1] Part II. E. CASPI and D. O. LEWIS, *Science* **214**, 519 (1967).

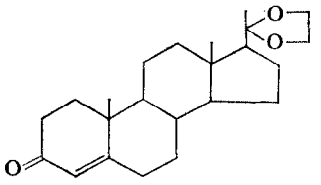
[2] This work was supported by Grants from U.S. Public Health Service (RO-1 HE-10566-02) and National Science Foundation (GB-5832). E. C. acknowledges the receipt of a Research Career Award (K3-16614).

[3] J. M. H. GRAVES and W. K. SMITH, *Nature* **214**, 1248 (1967).

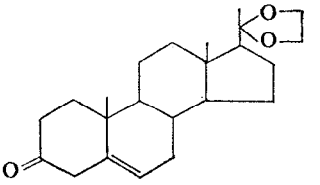
[4] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **6**, 1521 (1967).

RESULTS AND DISCUSSION

Progesterone-20-ethylene ketal (IV) was deconjugated with potassium *t*-butoxide in *t*-butanol by the method of Ringold and Malhotra.[5] The u.v. spectrum of the crude product shows that it contained *ca.* 78 per cent of the Δ^5-3-ketone (V) and *ca.* 22 per cent of the starting material. This mixture was reduced with sodium borotritide in diglyme solution to give the Δ^5-3β-OH compound together with some 20 per cent of the 3ξ-hydroxy-Δ^4-isomer. Acid treatment of this mixture removed the ketal and dehydrated the allylic alcohol (Δ^4-3ξ-OH) to a $\Delta^{3,5}$-diene, which was easily separated from the 3α-^{3}H-pregnenolone by thin-layer chromatography.



IV



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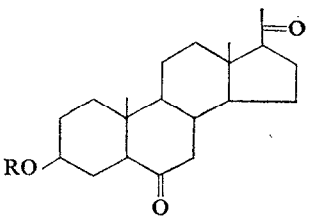


a. R=C_6H_5CO
b. R=H

VI

For determination of the distribution of label in the synthesized 3α-^{3}H-pregnenolone, the following reactions were carried out. Material recovered from base catalyzed equilibration of pregnenolone retained all the tritium showing that there was none at C_{17} or C_{21}. Hydroboration[6] of pregnenolone benzoate gave an intermediate which was oxidized with Jones' reagent to 3β-benzoyloxy-5α-pregnane-6,20-dione (VIa). Equilibration of the dione with base proceeded without loss of tritium proving the absence of label at C_6 and C_7. Catalytic hydrogenation[7] of pregnenolone provided a mixture of isomeric 5α-pregnane-3β,20ξ-diols which was oxidized with Jones' reagent. The resulting 5α-pregnane-3,20-dione (III) retained 13 per cent of the label. Oxidation at C_3 was shown to be complete by the fact that the tritium content of the dione was unaffected by a second treatment with the same reagent. Equilibration of the dione (III) with base gave a product devoid of tritium. Finally Oppenauer oxidation of pregnenolone and subsequent base equilibration removed all the tritium, confirming the above results. Thus 87 per cent of the label is at C_3, the remainder being distributed between C_2 and C_4 (Tables 1 and 2).

Doubly labeled 3α-^{3}H-4-^{14}C-pregnenolone, with an $^3H/^{14}C$ ratio of 13·3, was administered to the leaves of a young plant of *D. lanata*. Digitoxigenin (VII) (0·26 per cent incorporation based on ^{14}C) was isolated in the usual way[1, 8] after three weeks. The poor incorporation (usually, it is *ca.* 2 per cent) may be attributed to the fact that the experiment was conducted in February when the plant's rate of growth was low. The biosynthesized digitoxigenin had an $^3H/^{14}C$ ratio of 1·16 indicating that it retained about 9 per cent of the tritium present in the administered pregnenolone (Table 3). Oxidation to digitoxigenone (VIII) proceeded without loss of tritium, confirming the total absence of tritium at C_3 in the cardenolide. Hence the biosynthesis *must proceed* through the intermediacy of a 3-ketone.

[5] H. J. RINGOLD and S. K. MALHOTRA, *Tetrahedron Letters*, **15**, 609 (1962).
[6] The authors thank Dr. RAVI VARMA of this laboratory for carrying out this reaction.
[7] E. B. HERSHBERG, E. OLIVETO, M. RUBIN, H. STAEUDLE and L. KUHLER, *J. Am. Chem. Soc.* **73**, 1144 (1951).
[8] J. VON EUW and T. REICHSTEIN, *Helv. Chim. Acta* **37**, 711 (1964).

TABLE 1. DISTRIBUTION OF TRITIUM IN PREGNENOLONE

Compound	% ^3H retained	% ^3H at positions indicated	
Pregnenolone (I)	100		
Equilibrated pregnenolone	100	C_{17}, C_{21}	0
3β-Benzoyloxy-5-αpregnane-6,20-dione (VIa)	100	C_6	0
3β-Hydroxy-5α-pregnane-6,20-dione (VIb)	100	C_7	0
5α-Pregnane-3,20-dione (III)	13	C_3	87
Equilibrated 5α-pregnane-3,20-dione	0	C_2, C_4	13
Equilibrated progesterone (II)	0		

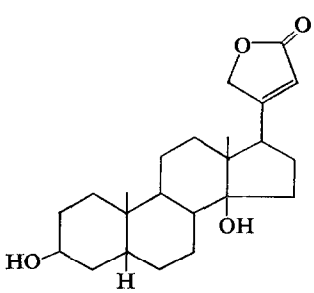
TABLE 2. DISTRIBUTION OF ^3H IN 6-KETO DERIVATIVES OF PREGNENOLONE ($\times 10^6$ dpm/mmole)

	1st crop	2nd crop	1st crop recrystal.	% ^3H retained
Pregnenolone	6·95		6·83	—
Pregnenolone benzoate	7·26	6·99		100
3β-Benzoyloxy-5α-pregnane-6,20-dione	6·84		6·97	100
3β-Hydroxy-5α-pregnane-6,20-dione	6·90		7·03	100

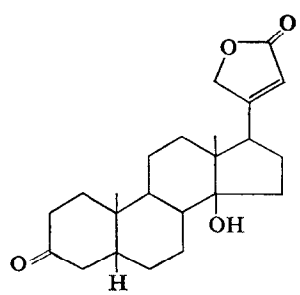
TABLE 3. $^3H/^{14}C$ RATIOS[a] OF BIOSYNTHESIZED DIGITOXIGENIN[b] AND ITS DEGRADATION PRODUCTS

	1st crystallization		2nd crystallization	
	1st crop	2nd crop	1st crop	2nd crop
Digitoxigenin (VII)	1·27		1·14	1·16
Digitoxigenone (VIII)	1·14	1·13		
Isodigitoxigenone (IX)	0[c]			

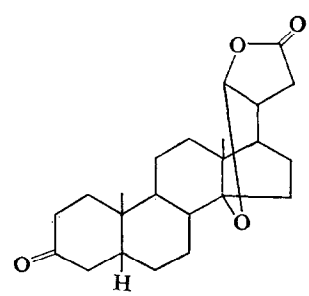
[a] Ratios based on dpm of ^3H and ^{14}C.
[b] Isolated after administration of pregnenolone ($^3H/^{14}C$ ratio 13·3) to *Digitalis lanata*.
[c] After purification as described in the text.



VII



VIII



IX

Equilibration of the labeled digitoxigenone (VIII) with base gave isodigitoxigenone (IX) devoid of tritium. The retained 9 per cent of label in digitoxigenin is therefore confined to C_2 and C_4.

EXPERIMENTAL

Melting points were determined on a hot stage and are corrected. Silica gel Merck HF_{254} was used for thin-layer chromatography (TLC). The samples were counted in a Nuclear Chicago Model 6860 Scintillation Counter. The scintillation fluid consisted of 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-[2-(5-phenyloxazoyl)]-benzene dissolved in 1000 ml of toluene.

Deconjugation of Progesterone-20-ethylene Ketal

A solution of progesterone-20-ethylene ketal (IV) (217 mg) in dry *t*-butanol (5 ml) was stirred with K *t*-butoxide (810 mg) under N_2 for 1½ hr at room temperature. The reaction was terminated by addition of aqueous acetic acid (25 ml of 90 per cent) and the dilute mixture was poured into $NaHCO_3$ (150 ml). The precipitated solid was collected, washed with water, and dried at 40°/0·1 mm. The u.v. spectrum of the solid, λ_{max}^{EtOH} 242 nm (ϵ, 3500) showed that it contained *ca.* 78 per cent of the Δ^5-3-ketone (V) and *ca.* 22 per cent progesterone-20-ethylene ketal. This mixture was used without further purification.

3α-^{3}H *Pregnenolone*

A solution of the Δ^5-3-ketone (V) (7·1 mg) in diglyme (0·3 ml) was treated with a diglyme solution of Na borotritide (0·74 mg 125 mC) and the mixture was left at room temperature for 22 hr. Water (0·15 ml) and 2 N HCl (0·3 ml) was added; after 1 hr the mixture was degassed. Water (1 ml) was added and the mixture worked up with ether. The product was dissolved in acetone and warmed with 2 N H_2SO_4 (0·1 ml) for 1 hr. After working up with ether the crude product was purified, first by TLC using 40 per cent ethyl acetate in benzene and then by continuous TLC (3 per cent methanol in benzene, 3 hr). In this way 3α-^{3}H-pregnenolone was effectively separated from the $\Delta^{3,5}$-diene and from progesterone. The resulting 3α-^{3}H-pregnenolone (6·94mC) was shown to be homogenous by paper chromatography (Bush A) and by cocrystallization of an aliquot with nonradioactive material to constant specific activity (crude: $2·15 \times 10^6$ dpm/mmole. 1st and 2nd crops: $2·13 \times 10^6$ dpm/mmole).

This diluted 3α-^{3}H-pregnenolone was equilibrated by refluxing under N_2 with 3 per cent KOH in 70 per cent aqueous methanol for 2½ hr. The pregnenolone so obtained was recrystallized from ethyl acetate and had the same activity ($2·15 \times 10^6$ dpm/mmole) as the starting material.

Oppenauer oxidation of the diluted 3α-^{3}H-pregnenolone gave progesterone (II), which was devoid of radioactivity after base equilibration as above.

5α-*Pregnane*-3,20-*dione* (*III*)

3α-^{3}H-pregnenolone ($2·38 \times 10^6$ dpm/mmole, 5 g) was hydrogenated in ethyl acetate solution with Adams catalyst (0·2 g) and 72 per cent $HClO_4$ (0·15 ml) at room temperature for 36 hr. Filtration and evaporation afforded a mixture of 5α-pregnane-3β,20ξ-diols ($2·38 \times 10^6$ dpm/mmole).

Jones' oxidation of this diol for 10 min at room temperature gave crude 5α-pregnane-3,20-dione(III) ($3·11 \times 10^5$ dpm/mmole, 13 per cent of ^{3}H retained). No further loss of radioactivity was observed after a second 10-min oxidation of this material ($3·04 \times 10^5$ dpm/mmole). A sample of the dione was crystallized to a m.p. 193–6° (lit.[9] m.p. 200·5°).

Prolonged equilibration (12 hr) of the dione and recrystallization (ethyl acetate) gave 5α-pregnane-3,20-dione which was devoid of radioactivity.

3β-*Hydroxy*-5α-*pregnane*-6,20-*dione* (*VIb*)

To a solution of pregnenolone benzoate (1·05 g, 2·5 mmoles) in dry tetrahydrofuran was added a solution of B_2H_6 in tetrahydrofuran (6·6 ml of 0·37 M in B_2H_6) during 5 min with stirring and ice cooling. The mixture was stirred overnight in the cold room, and excess hydride then decomposed by careful addition of water. After evaporation to dryness at about 45° *in vacuo* ether (30 ml) and benzene (30 ml) were added. A solution of chromic acid prepared from $Na_2Cr_2O_7\,2H_2O$ (440 mg), water (10 ml) and conc. H_2SO_4 (0·33 ml) was added slowly at 0° with vigorous stirring. After 45 minutes at 0°, the organic layer was separated and the aqueous layer extracted several times with ether. The combined organic layers were washed with $NaHCO_3$ solution and water, dried (Na_2SO_4) and evaporated to give a solid, ν_{max} (KBr) 1710, 1685 cm^{-1}. Two recrystallizations from ethyl acetate-methylene chloride afforded 3β-*benzoyloxy*-5α-*pregnane*-6,20-*dione* (VIa, 0·55 g), m.p. 215–216°; mass spectrum showed M^+ at *m/e* 436; infrared and NMR spectra were consistent with this structure.

Refluxing the keto-benzoate with 5 per cent KOH in 80 per cent aqueous methanol provided the hydroxy-dione (VIb). This was oxidized by Jones' reagent to 5α-pregnane-3,6,20-trione, which was crystallized from acetone, m.p. 230–232° (lit.[10] m.p. 232–233°).

The above series of reactions were carried out on a sample of diluted 3α-^{3}H-pregnenolone. The individual compounds were crystallized from ethyl acetate and counted (Table 2).

[9] *Elsevier's Encyclopaedia of Organic Chemistry*, Elsevier, New York 1940, Vol. 14. The reported m.p. for 5β-pregnane-3,20-dione is 123°.

[10] C. P. BALANT and M. EHRENSTEIN, *J. Org. Chem.* **17**, 1587 (1952).

Administration of 3α-^{3}H-4-^{14}C-*Pregnenolone to Digitalis lanata; Isolation of Digitoxigenin*

3α-^{3}H-^{14}C-pregnenolone ($2{\cdot}35 \times 10^{7}$ dpm of ^{14}C, ^{3}H/^{14}C ratio 13·3) was administered to all but one of the leaves of a whole young plant of *D. lanata*, as previously described.[1]

After 3 weeks the plant was harvested and digitoxigenin isolated as previously described.[1,8] It was necessary to submit the material to a fourth chromatography (Whatman's No. 1, benzene:methanol:water—2:1:1) in order to obtain homogenous digitoxigenin ($6{\cdot}03 \times 10^{4}$ dpm of ^{14}C, 0·26 per cent incorporation). This was diluted with 40 mg of nonradioactive compound and crystallized from ethyl acetate to a constant ^{3}H/^{14}C ratio (Table 3).

Degradation of Digitoxigenin (VII)

Diluted digitoxigenin (1st crop, 13 mg) was oxidized for 5 min with Jones' reagent (0·05 ml) in acetone (1 ml) at 10°. The reaction mixture was poured into $NaHCO_3$ solution and extracted with ethyl acetate. The product crystallized from ethyl acetate to give digitoxigenone (VIII), m.p. 200° (lit.,[9] m.p. 202–3°) (Mass spectrum: M^+ at *m/e* 372) of the same activity as the starting material (Table 3).

The mother liquors from crystallization of digitoxigenone (homogenous by TLC) were evaporated and refluxed 12 hr with 5 per cent KOH in 80 per cent aqueous-methanol (5 ml). The reaction mixture was concentrated under N_2 at 40° and acidified to Congo Red with 2N HCl. Extraction with ethyl acetate gave isodigitoxigenone (IX) which was purified by continuous TLC (40 per cent ethyl acetate in benzene, 3 hr). Three crystallizations from ethyl acetate gave needles m.p. 249–251° (lit.,[9] m.p. 264°); mass spectrum showed M^+ at *m/e* 372; the ^{3}H/^{14}C ratio was zero.